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Short-Term Intraindividual Variability in Plasma Trace Mineral Concentrations

Noel W. Solomons,* Oscar Pineda* & David B. Milne†

* Division of Nutrition and Health, Institute of Nutrition of Central America and Panama, Carretera Roosevelt, Zona 11, Guatemala City, Guatemala AP 11-88.
† United States Department of Agriculture Grand Forks Human Nutrition Research Center, Grand Forks, North Dakota, USA

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ABSTRACT

Serial fasting samples of blood plasma were analyzed by atomic absorption spectrophotometry for concentrations of zinc, copper and iron in 10 subjects on five occasions (Series A) and in another 10 subjects on three occasions (Series B) during a 2–3 week period. The intrasubject variability, estimated by coefficients of variation for the mean of repeat determinations, ranged from 0·6 to 15·6% for zinc, 1·1 to 15·9% for copper, and 3·5 to 43·2% for iron. The respective coefficients of variation for instrumental variability within an analytical run were all less than 3%. Application of a tourniquet for venous occlusion prior to venipuncture could account for some of the additional variance. Nonvisible microhemolysis could have contributed to the larger variance in serial iron determinations. We conclude that much of the day-to-day intrasubject variation was due to true biological changes in the distribution of trace metals. Fluctuations in fasting trace metal concentrations have important implications for their use as indices of nutritional status.

Key words: trace metal nutriture, zinc, copper, iron, atomic absorption spectrophotometry, biological variation.

The advent of atomic absorption spectrophotometry has permitted rapid, precise and accurate determination of circulating levels of trace minerals. It has contributed to a rapid expansion in our

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understanding of human trace mineral metabolism (Reinhold, 1975; Mertz, 1981). Determination of circulating mineral levels has been used to assess trace mineral nutriture of both populations and individuals (Solomons, 1979). When a given trace mineral concentration is used as a diagnostic reference in a given subject, however, the intraindividual variability of that measurement is an important consideration. Recently, in the context of multiple, randomized zinc absorption tests using the change in plasma zinc as the index of uptake, we were able to measure fasting trace mineral concentrations repeatedly in the same individuals during a short interval of time (2–3 weeks). The results provide an insight into the reproducibility of the measurements in the same individual.

MATERIALS AND METHODS

Two series of experiments were performed, each with 10 subjects. In Series A, the participants were sampled on *five* distinct occasions during a period of from 14 to 21 days. This group included eight women and two men, ranging in age from 18 to 47 years (mean: 31 years). In Series B, subjects were tested on *three* separate occasions during a period of up to 14 days. In this group, there were seven females and three males, aged 26 to 48 years (mean: 33 years). Five individuals were common to both groups, but, since the two studies were displaced in time by several months, they have been treated as independent series.

On each morning of a test, the subjects would arrive at the laboratory between 7.30 and 8.30 a.m. having fasted. A sample of 4 to 5 ml of venous blood was drawn from an antecubital vein through a stainless steel needle into a plastic syringe with a rubber tourniquet applied to the arm for temporary venous occlusion. The sample was transferred to a trace-mineral-free plastic tube containing 0·05 ml of a 20% potassium oxalate solution as anticoagulant, centrifuged, and the plasma separated and stored in another plastic tube under refrigeration until analysis.

Plasma samples were diluted 1:5 with distilled, deionized water, and analyzed by atomic absorption spectrophotometry. All samples from a given series were analyzed in a single run on a single day, and standard pooled sera were analyzed frequently at intervals within the

run. Samples with *visible* hemolysis were identified and the iron analyses from such samples were discarded. Only one fasting sample in each of the series was observed to have detectable hemolysis (pink plasma) by the unaided eye. The concentrations of the trace metals in plasma were expressed as micrograms per deciliter.*

RESULTS

Analytical reproducibility

For each metal, two reference plasmas, one with a high and the other with a low concentration, were measured at intervals during the analytical run. The coefficients of variation (CVs) for the intra-run reference plasmas for the series were calculated to determine the fraction of the observed variance that might be attributed to measurement (analytical) error. For Series A, the repetitions of the control plasma for zinc ($n = 5$) yielded intra-run CVs of 1·0 and 2·7% for the high-metal and low-metal plasma, respectively; for copper ($n = 6$) the respective intra-run CVs were 1·5 and 2·1%; and for iron ($n = 6$) the respective intra-run CVs were 2·4 and 2·7%. The findings were comparable for Series B.

Biological (intraindividual) variation

The results for means, standard deviations, coefficients of variation and differences between the highest and lowest recorded metal concentrations are shown for zinc (Table 1), copper (Table 2), and iron (Table 3) for the two series. The CVs for five serial plasma zinc determinations (Series A) ranged from 2·8 to 15·6%, and for three serial determinations (Series B) ranged from 0·6 to 10·0%. The least high–low difference in zinc concentration in Series A was 7 $\mu g\,dl^{-1}$, and in Series B 1 $\mu g\,dl^{-1}$. The corresponding maximum high–low differences were 31 and 15 $\mu g\,dl^{-1}$ in the respective series (Table 1).

For plasma copper determination, the CVs ranged from 1·1 to 14·5% for Series A, and 1·6 to 15·9% for Series B. Minimum and

* For plasma zinc, 1·0 μ mol $dl^{-1} = 65{\cdot}4\ \mu g\,dl^{-1}$; for plasma copper, 1·0 μ mol $dl^{-1} = 63{\cdot}6\ \mu g\,dl^{-1}$; for plasma iron, 1·0 μ mol $dl^{-1} = 55{\cdot}8\ \mu g\,dl^{-1}$.

TABLE 1
Values and Descriptive Statistics for Serial Fasting Plasma Zinc Concentrations Five Occasions in 10 Subjects (Series A) and on Three Occasions in 10 Subjects (Series B)

	Plasma zinc concentration ($\mu g\ dl^{-1}$)									
Series A subjects	*01*	*02*	*03*	*04*	*05*	*06*	*07*	*08*	*09*	*10*
	112	95	83	74	120	88	70	80	116	90
	99	93	81	88	90	90	88	71	107	67
	104	99	71	84	112	90	86	67	95	82
	83	95	74	95	112	101	80	64	89	87
	104	92	104	104	121	100	97	74	99	86
$\bar{x}$	100·4	94·8	82·6	89·0	111·0	93·8	84·2	71·2	101·2	84·2
SD	10·8	2·7	12·9	11·3	12·4	6·2	10·0	6·2	10·5	9·1
CV (%)	10·7	2·8	15·6	12·7	11·2	6·6	11·9	8·7	10·4	11·0
Δ	29	7	21	30	31	13	27	16	21	23

	Plasma zinc concentration ($\mu g\ dl^{-1}$)									
Series B subjects	*001*	*002*	*003*	*004*	*005*	*006*	*007*	*008*	*009*	*010*
	63	95	92	80	91	81	80	95	96	73
	77	90	85	87	91	84	90	84	90	84
	69	83	90	84	92	74	85	87	85	80
$\bar{x}$	69·7	89·3	89·0	83·7	91·3	79·7	85·0	88·7	90·3	79·0
SD	7·0	6·0	3·6	3·5	0·6	5·1	5·0	5·7	5·5	5·6
CV (%)	10·0	6·7	4·0	4·2	0·6	6·4	5·8	6·4	6·1	7·0
Δ	14	12	7	7	1	10	15	8	11	11

maximum intraindividual high–low differences were 2 and 66 $\mu g\ dl^{-1}$ in the former series, and 4 and 23 $\mu g\ dl^{-1}$ in the latter series (Table 2).

For plasma iron, the CVs in the five-sample series varied from 9·0 to 34·5%, and the minimum and maximum high–low differences were 25 and 129 $\mu g\ dl^{-1}$, respectively (Table 3). In the three-sample series, the CVs ranged from 3·5 to 43·2%, and the minimum and

TABLE 2
Values and Descriptive Statistics for Serial Fasting Plasma Copper Concentrations Five Occasions in 10 Subjects (Series A) and on Three Occasions in 10 Subjects (Series B)

	Plasma copper concentration ($\mu g\ dl^{-1}$)									
Series A subjects	*01*	*02*	*03*	*04*	*05*	*06*	*07*	*08*	*09*	*10*
	109	142	129	110	184	99	92	85	83	112
	106	152	121	105	148	94	97	83	103	114
	104	152	118	99	216	97	97	83	90	116
	104	151	115	108	214	90	70	83	101	110
	108	138	114	135	196	92	90	83	94	110
$\bar{x}$	106·2	147·0	119·4	111·4	191·6	94·4	89·2	83·4	94·2	112·4
SD	2·2	6·6	6·0	13·8	27·7	3·6	11·1	0·9	8·2	2·6
CV (%)	2·2	4·5	5·1	12·4	14·5	3·9	12·5	1·1	8·7	2·3
Δ	5	14	15	36	66	9	27	2	13	6

	Plasma copper concentration ($\mu g\ dl^{-1}$)									
Series B subjects	*001*	*002*	*003*	*004*	*005*	*006*	*007*	*008*	*009*	*010*
	125	121	123	148	86	103	133	115	111	100
	111	107	121	164	84	100	127	119	82	100
	102	119	110	158	91	90	121	102	90	104
$\bar{x}$	112·7	115·7	121·0	156·7	87·0	97·7	127·0	112·0	94·3	101·3
SD	11·6	7·6	2·0	8·0	3·6	6·8	6·0	8·9	15·0	2·3
CV (%)	10·3	6·5	1·6	5·2	4·1	6·9	4·7	7·9	15·9	2·3
Δ	23	14	4	10	7	23	12	17	21	4

maximum high–low differences were 8 and 75 $\mu g\ dl^{-1}$, respectively (Table 3).

DISCUSSION

The short-term variability of serial determinations of serum or plasma trace metal concentrations has not been extensively studied.

TABLE 3
Values and Descriptive Statistics for Serial Fasting Plasma Iron Concentrations Five Occasions in 10 Subjects (Series A) and on Three Occasions in 10 Subjects (Series B)

	Plasma iron concentration ($\mu g\ dl^{-1}$)									
Series A subjects	*01*	*02*	*03*	*04*	*05*	*06*	*07*	*08*	*09*	*10*
	142	105	95	97	255	101	142	77	190	155
	131	98	146	72	—	90	158	70	154	95
	175	89	81	74	221	99	180	81	147	126
	181	80	108	101	126	111	151	99	155	128
	188	89	140	120	135	118	155	72	118	154
$\bar{x}$	163·0	92·2	114·0	92·8	184·2	103·8	157·2	79·8	152·8	131·6
SD	25·2	9·6	28·2	20·1	63·7	10·9	14·1	11·6	25·7	24·7
CV (%)	15·5	10·3	24·7	21·6	34·5	10·5	9·0	14·5	16·8	18·7
Δ	55	25	65	48	129	28	38	29	72	60

	Plasma iron concentration ($\mu g\ dl^{-1}$)									
Series B subjects	*001*	*002*	*003*	*004*	*005*	*006*	*007*	*008*	*009*	*010*
	140	76	68	189	132	84	59	137	116	97
	76	91	70	143	111	86	103	154	113	162
	65	86	94	132	111	62	51	124	121	170
$\bar{x}$	93·7	84·3	77·3	154·7	118	77·3	71·0	138·3	116·7	143·0
SD	40·5	7·6	14·5	30·2	12·1	13·3	28·0	15·0	4·0	40·0
CV (%)	43·2	9·1	18·7	19·6	10·3	17·2	39·4	10·9	3·5	28·0
Δ	75	15	26	57	21	24	52	30	8	73

In one early report, Vikbladh (1951) obtained fasting, morning blood samples for zinc analysis on 2 consecutive days in four subjects. He used a colorimetric (dithizone) procedure for quantification of serum zinc. The differences in serum zinc between the first and second day were 10, 4, 6 and 4 $\mu g\, dl^{-1}$, respectively. Oelshlegel and Brewer (1977), in the context of repeat zinc tolerance tests performed over a year in nine subjects at 5–11 different times, calculated intra-individual means and standard deviations. Estimating from their graphic display of data, CVs ranged from 5 to 15%. Our global CV

for zinc, as a composite mean from our two series, was 7·9 ± 3·7% (±SD).

Vallee (1952) analyzed serum copper on two consecutive mornings in one subject. The levels differed by 7 $\mu g\,dl^{-1}$. In another subject, fasting samples were collected on four mornings during a 7-day period. The CV was 3·6%. In yet another subject, 13 samples of blood taken at approximately 10 a.m., some in the fasting state but usually in the fed state over an interval of a month, showed a CV of 3·7%. Thirty per cent of our subjects in the present 14- to 21-day experience had CVs equal to or less than the Vallee (1952) values. Cartwright and Wintrobe (1964) actually recorded nine serum copper determinations on a single subject over an 11-year period from 1951 to 1963. The CV was 22·9%. Our data here, and those of Vallee (1952), would suggest that short-term (over a month) stability of circulating copper concentrations is much greater than long-term stability, measured over a decade (Cartwright & Wintrobe, 1964).

For iron, serial data on fasting circulating concentrations come from three publications (Hoyer, 1944; Statland *et al.*, 1976; Statland & Winkel, 1977). In the classical study by Hoyer in 1944, eight male and 12 female subjects were studied at weekly intervals for 2 to 3 months. Each subject had 8–15 individual determinations. The respective coefficients of variation were 22·5 and 25·9% for men and women. Statland and co-workers found a CV for four serial iron determinations in 11 men, taken at weekly intervals, of 26·6% (Statland *et al.*, 1976) and a CV of 29·3% for 27 determinations over a 4·4 month interval in nine women (Statland & Winkel, 1977). These compare to an overall combined CV for plasma iron for our Series A and B of 18·3 ±11·2%. In the Hoyer (1944) series, the difference between the highest and lowest serum iron for a given individual ranged from 69 to 134 $\mu g\,dl^{-1}$. One of our subjects (05) had a span in iron concentrations of 129 $\mu g\,dl^{-1}$.

As the foregoing comparative review illustrates, literature on intraindividual variability in trace metal concentrations is scarce, and much of it derives from colorimetric analytical procedures. Only the zinc in the study by Oelshlegel & Brewer (1977) and the iron from the Statland & Winkel (1977) series were measured by atomic absorption spectrophotometry (AAS). AAS generally provides greater measurement precision as well as speed and efficiency in analyses. It also conveniently allows the determination of *several* trace metals

on a single dilution of serum or plasma. It was for this reason that we could readily perform the triad of determinations on the serial fasting samples of blood plasma collected in two experimental series in our laboratories. It is revealing that both the present series and all of the available literature reviewed report large intraindividual variability for concentrations of zinc, copper and especially iron.

Measurement error in our determinations (see above) can account for only a small portion of the variance. We analyzed all of our samples from a given series on the same day, and the intra-run CVs were no greater than 3% for any metal, even in low abundance. Similarly, in all of the previous literature (Hoyes, 1944; Vikbladh, 1951; Vallee, 1952; Cartwright & Wintrobe, 1964; Statland *et al.*, 1976; Oelshlegel & Brewer, 1977; Statland & Winkel, 1977), intraindividual variation was consistently greater than analytical variation no matter what approach to quantification, colorimetric or AAS, was used. Another possible source of variability, here and elsewhere, is the application of a tourniquet during the extraction of the blood sample. We have previously shown that venous occlusion during venipuncture significantly influences (raises) the concentrations of zinc (mean increment, $2{\cdot}5 \pm 4{\cdot}5\,\mu g\,dl^{-1}$) and iron (mean increment, $18{\cdot}5 \pm 15{\cdot}0\,\mu g\,dl^{-1}$), but not copper, as compared to blood from an unoccluded vein (Juswigg *et al.*, 1982). In the present study, a tourniquet was consistently applied to the arm while obtaining samples. Thus, variability in the duration and pressure of application could account for a further small degree of the total variance in zinc and iron concentrations observed.

It is noteworthy that plasma iron has the greatest amount of variation, on average twice that of zinc or copper. Our blood technician exhibited a high degree of skill in obtaining suitable samples as evidenced by the appearance of *visible* hemolysis in only two of 80 plasma specimens. Nonetheless, we can speculate that nonvisible *micro*hemolysis of samples could account for the excess variability in the determinations of plasma iron as compared to the other two metals. The discrimination concentration between a visibly hemolyzed and non-hemolyzed serum sample is in the order of $20\,mg\,dl^{-1}$, corresponding to $68\,\mu g\,dl^{-1}$ of heme-iron. AAS would not discriminate heme-iron from iron bound to transferrin and other plasma carriers. Jacobs and Worwood (1978) have commented incisively on the role of AAS determination of plasma iron: 'Despite

the sensitivity of modern atomic absorption spectrophotometry, colorimetric methods are still generally preferred because of the necessity to measure only transferrin iron and avoid any contribution from heme iron present as a result of hemolysis.' Indeed, international standardized procedures for determination of serum iron involve the trichloroacetic acid precipitation of protein to eliminate from analysis any tightly bound (hemoglobin) iron that might be derived from incidental hemolysis of the sample (International Committee for Standardization in Hematology, 1971). Thus, undetected microhemolysis could have accounted for some of the additional error. In the literature reviewed (Hoyer, 1944; Statland *et al.*, 1976; Statland & Winkel, 1977), the methods for iron determination did not adequately eliminate the contamination of serum by heme-iron, and they share a common vulnerability with our own series to artifactual elevations of iron levels due to microhemolysis.

Although the instrument and the tourniquet might account for some, and microhemolysis for yet more with respect to iron and zinc, the majority of the variability in plasma trace metals in our series must be attributed to *biological* (individual) bases. It is known that all three metals have a circadian variation. Although our samples were taken at the same time of day and in the fasting state, differences in periodicity of the diurnal cycle could account for some day-to-day variation. Subjects were healthy and consuming their normal diets. Conceivably, however, some variation in the state of hydration or some persistence of the direct effect of a previous day's meal could also represent factors altering blood mineral concentrations. Furthermore, all three minerals are influenced by hormonal factors. Leukocytic endogenous mediator(s) (LEM) from activated white cells are capable of reducing the concentrations of zinc and iron, while raising the concentrations of copper (Powanda & Beisel, 1982). Despite freedom from apparent illness on the days of sampling, the microbiotic environment of Guatemala could have produced subclinical gastrointestinal bacterial colonization or infection in our subjects, modulating LEM production. Estrogens influence ceruloplasmin concentration (Johnson *et al.*, 1959), and the variance in copper levels in our female subjects, constituting 75% of our population, might have been influenced by the hormonal changes of the menstrual cycle. Endogenous corticosteroids can also reduce concentrations of zinc (Henkin *et al.*, 1965), and possibly those of

copper (Yunice *et al.*, 1981). Faced with data from several determinations of plasma trace metal concentrations for the assessment of nutritional status, the conservative approach might be to take the lowest value, on the assumption that contamination accounted for the higher values. With the variety of *endogenous* factors that can modify zinc, copper and iron concentrations, however, such a conclusion may not be justifiable. Aside from errors introduced in sampling, handling and analysis, each determination here may have represented the 'true' and accurate level of circulating mineral on the given morning on which it was obtained, with *biological* day-to-day fluctuations in fasting trace metal concentrations being the major source of overall variance.

With respect to iron, the advice of Jacobs & Worwood (1978) (see above) appears to be timely and enduring, for despite the simplicity of AAS for iron determination, the more formal, standard, internationally recognized procedures involving precipitation of hemoglobin and colorimetry are still required for valid iron measurements. Limitations in the application of circulating levels of zinc and copper to the assessment of nutritional status have been discussed (Solomons, 1979); the degree and magnitude of short-term variability in plasma copper and zinc observed in the present series provide yet another reinforcing argument to our admonition against a total reliance on circulating metal levels for the estimation of human trace mineral status.

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