Zinc Determined in 10-μL Serum or Urine Samples by Flameless Atomic Absorption Spectrometry

Nancy E. Vieira and James W. Hansen

We describe a precise flameless atomic absorption spectrometric method requiring only 10 μL of sample, which thus permits repeated measurements in the neonate. Standard curves covering the range of 0 to 30 mg of zinc per liter, with standards in various matrices (albumin, glycerol, serum, or nitric acid) had slopes ranging from 0.49 to 1.17, relative to that for aqueous standards. We prepared low-zinc matrices, which had slopes similar to that of serum, by dialyzing serum or a 50 g/L solution of albumin vs a buffered zinc-free dialysis fluid containing appropriate inorganic constituents. Use of thoroughly de-ionized water, reagent-grade chemicals, "ultrapure" nitric acid, specified disposable plastic ware, and appropriate pipet tip-rinsing techniques minimized extraneous contamination with zinc. Concentrations of zinc in serum calculated from a "rational-method" calibration algorithm fit to the standard curve agreed well with independent determinations by flame atomic absorption spectrometry.

Additional Keyphrases: albumin, glycerol, serum, and nitric acid matrices compared • neonatal chemistry • variation, source of • nutritional status • trace elements • reference intervals

Information concerning the concentration of zinc in body fluids has become increasingly important because the role of zinc in both normal and abnormal growth and development is better recognized. In addition, oral and parenteral dietary supplements that include zinc are being used more frequently in sick and undernourished patients, including premature infants. In the case of newborns, the current sample volumes required for analysis may produce hematological problems for the patient, which discourages measurements at appropriately frequent intervals to monitor mineral status and nutritional adequacy.

The high sensitivity and specificity of flameless atomic absorption spectrometry permitted us to develop a convenient and reliable technique for measuring zinc in 10 μL of sample.

Materials and Methods

Apparatus

All determinations were made with a Model 5000 atomic absorption spectrometer equipped with an HGA 2100 graphite furnace with ramp (gradual temperature increase) accessory and a Model 056 strip-chart recorder (all from Perkin-Elmer Corp., Norwalk, CT 06856). A Perkin-Elmer hollow-cathode zinc lamp was used as the source, at a current of 15 mA.

The spectrometer was operated at 213.9 nm, in the peak height mode, and with a 0.7-nm low slit width. Graphite furnace conditions, established according to Fernandez and lannarone (1), were: dry for 60 s with 10-s ramp to 95 °C, char for 30 s with 15-s ramp to 450 °C, and atomize for 6 s at 2400 °C.

Argon was used as the purge gas flooding the graphite furnace. We adjusted the flow rate to 60 flow-meter divisions (corresponding to 110 mL/min) during atomization, to reduce zinc sensitivity threefold. This avoided excessive dilution of the sample, which can result in significant extraneous zinc contamination. The final sensitivity for a typical zinc assay at these settings is 37 pg/0.1 absorbance unit (A).

Reagents

All water used in these studies was processed through de-ionizers manufactured and maintained by Hydroservice and Supplies, Inc., Durham, NC 27705; it then contained <15 ng of zinc per liter (<0.4 μA/10 μL). All chemicals used were analytical-reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) unless otherwise noted. "Ultrapure" nitric acid was used in standard curve and sample preparation (J. T. Baker, "Ultrex" grade). "Certified Atomic Absorption Reference Solution" containing 1 g of zinc per liter (Fisher Scientific Co., Fair Lawn, NJ 07410) was used to prepare the standard curve. The albumin used in the procedure was Bovine Albumin No. A-4-378 (Sigma Chemical Co., St. Louis, MO 63178).

Procedures

Wherever possible, disposable plastic ware was used to collect specimens and to prepare them for analysis. Moody and Lindstrom (2) and others (3) have tested various plastics and found conventional polyethylene and polypropylene containers to be essentially free of zinc. Rubber plungers and stoppers were invariably contaminated with zinc (3-6). To collect blood samples, we used Monoject syringes and needles (4) (Sherwood Medical Industries, Inc., Deland, FL 32720). Rinsing the syringes and needle combinations in de-ionized water increased zinc concentrations by less than 5 μg/L (0.5% of normal serum values).

Blood samples were allowed to clot in Falcon labware tubes (nos. 2059 and 2063; Falcon, Oxnard, CA 93030) and the serum was stored in the same type of tubes. We also used these tubes for all sample dilutions and solutions used in preparing

---

1 We did not treat any of the samples with trichloroacetic acid because of the potential for zinc contamination from such reagents.

Neonatal and Pediatric Medicine Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20205.

Received May 9, 1980; accepted Sept. 10, 1980.
standard curves. De-ionized water stored in the tubes for several days did not acquire any detectable zinc from them. To prepare large volumes of zinc-free solutions, glassware was immersed in 1.6 mol/L nitric acid for 24 h, rinsed four times with water, and allowed to dry in a basket that was lined and covered with absorbent paper not shown to contaminate the glassware with zinc. Such glassware was handled only with plastic gloves that first were rinsed in nitric acid and then in water to avoid contamination from the relatively high amounts of zinc present on the surface of the skin. A new Monoject needle was used to free the clot from the tubes, thus avoiding zinc contamination from wooden applicators. Disposable Eppendorf, tray-mounted, polypropylene micropipet tips, supplied in covered boxes of 100 tips, were used to dilute samples, to prepare standard curves, and to introduce specimens into the graphite furnace. To eliminate a small but variable degree of zinc contamination from these tips, immediately before use we rinsed each tip twice with an 0.8 mol/L "ultrapure" HNO₃ solution and then three times with water in three different plastic tubes (to minimize contamination from repetitive rinsing in a single tube), a procedure we found to be quick and adequate.

Serum was diluted 100-fold by adding 10 μL of serum to 990 μL of water; 10 μL of this solution gave readings of about 0.2 A. To obtain complete and reproducible delivery of dilutions of serum, a rinsed pipet tip was filled and the outside carefully dried with a paper wipe to prevent the aliquot from sticking to and climbing up the tip upon expulition as a result of the surface-tension-reducing properties of protein solutions.

We introduced 10-μL samples into the furnace, using a new, rinsed pipet tip for each sample. Three identical dilutions of each serum sample were used to provide three readings. The dilution resulting in the median reading was then assayed twice more and the median of the three readings on this dilution was regarded as the most precise estimate of the true serum value.

Matrix Effects in Serum or Plasma

Matrix effects were observed when the slope of the linear portion of a standard curve prepared with aqueous standards was found to be greater than the slope of a serum specimen to which known amounts of zinc were added. Standard curves were prepared by use of 100-fold dilutions of the matrixes shown in Table 1. Various amounts of zinc were added to increase the final Zn concentration by 0, 3, 5, 10, 20, and 30 μg/L. Pooled serum, plasma, or albumin solutions were dialyzed to decrease their zinc content so they could be used in preparing standard curves. The dialyzing fluid was made up to contain the following: Na⁺ (144 mmol/L), K⁺ (5.0 mmol/L), Cl⁻ (108 mmol/L), HCO₃⁻ (25 mmol/L), C₂H₃O₂⁻ (25 mmol/L), inorganic phosphate (53 mmol/L), dextrose (1 g/L), Ca²⁺ (500 mg/L), Mg²⁺ (20 mg/L). Tris buffer (3 mmol/L); the pH was 7.3. Sodium citrate (10 mmol/L) was added for dialyzing citrated specimens.

The solution to be dialyzed, containing 2 mmol of EDTA per liter, was placed in a 1-μm Visking Brand cellulose dialysis bag. The dialysis bag was prepared for use by soaking it in de-ionized water for at least 1 h, then dipping it several times in 1.6 mol/L "ultrapure" nitric acid, and finally rinsing well with de-ionized water. Dialysis was for 3 to 4 h, in an acid-washed graduated cylinder; the dialysis fluid was then changed and dialysis continued overnight. By leaving several milliliters of air in the dialysis bag and tying it so that there was slight pressure inside, the specimen weight did not change appreciably. The zinc reference standard was first diluted 10 000-fold with de-ionized water. Aliquots of the resulting 100 μg/L standard were then used to prepare a series of standards containing 0, 3, 5, 10, 20, and 30 μg of zinc per liter of the diluted matrixes.

Calculations

To account for the zinc remaining in the dialyzed specimens, we performed a linear regression over the linear portion of the standard curve and determined the concentration intercept as in the method of additions. We then added this value, typically 0.8 to 2 μg/L, to the concentration of zinc in the standard and used the calculated total zinc content to determine the smooth standard curve by the method of least squares.

The zinc standard curve became nonlinear just above typical serum values; further dilution decreased accuracy and precision. To compensate for the nonlinearity, we used the "rational-method" calibration algorithm proposed by Limbeck et al. (7) to arrive at a smooth standard curve from which to interpolate the specimen concentrations. We found that a two-parameter curve was necessary and sufficient to fit the data. To incorporate multiple points on the standard curve, we derived the following least-squares equation to fit the data:

$$c = A / (P + QA)$$

where c = concentration
A = absorbance units
P = \( \frac{[\sum c^{2}A^{2} + \sum cA]}{[\sum c^{2}A^{2} \sum c^{2} - (\sum c^{2}A^{2})]} \)
Q = \( \frac{[\sum c^{2}]}{[\sum c^{2}A^{2}]} \)

These equations are readily programmed into a small desk-top programmable calculator, facilitating the calculation.

Evaluation of Method

To evaluate our method, we measured the zinc content of unknown plasma samples previously analyzed by flame atomic absorption spectrometry techniques (8) in another laboratory (Children's Hospital National Medical Center, Washington, DC).

Results

Table 1 lists the slopes of the standard curve in the various matrixes, expressed as a fraction of the slope in de-ionized water. Dialyzed albumin (50 g/L), serum, or plasma appeared
to provide appropriate and interchangeable matrices, but all have significantly shallower slopes than water or nitric acid matrices. Slopes for 60 and 125 g/L solutions of glycerol also differed from that for albumin solutions. The slopes for the dialysis fluid preparation as matrix and water-dialyzed 50 g/L albumin matrix are markedly less than those of the other matrices. Matrix effects on urine zinc varied from specimen to specimen and therefore a single standard curve could not be used. Urinary zinc could be determined only by the method of additions at a 100-fold dilution.

Figure 1 shows standard curves prepared by using nitric acid, water, albumin, and glycerol matrices. The least-squares fit of the "rational-method" calibration algorithm was shown to fit the data precisely; few data deviated from the calculated curve. The slope of the curve decreased with the matrix used, in the order nitric acid > water > albumin > glycerol.

For zinc in plasma or serum, analysis of variance showed within-assay variation to be 3.3% and between-assay variation to be 16% with dialyzed serum or albumin as the matrix used in preparing the standard curve. Zinc values obtained by our technique were compared with those obtained by conventional flame atomic absorption spectrometry. The means of the groups of samples (n = 36) did not differ significantly: 0.77 and 0.81 mg/L, respectively. The standard deviation for zinc values in the samples measured in the graphite furnace (0.14 mg/L) was slightly less than that for those measured by flame (0.18 mg/L), resulting in a narrower reference interval. However, for individual paired samples, the values determined by the graphite furnace were consistently lower and thus the mean difference was 5% lower (p <0.05). The coefficient of correlation between the two methods was 0.90, with a regression line defined by: graphite furnace value = 0.975 X flame value; the slope of this line is not significantly different from the ideal value of unity (Figure 2).

From the zinc-recovery experiments, recovery of 0.83 μg/L added zinc was 0.85 μg/L (101% ± 0.016 SE (n = 46)) and of 1.67 μg/L added zinc was 1.70 μg/L (102%) ± 0.029 SE (n = 43).

Zinc concentrations were measured in sera of 105 clinically healthy subjects at the NIH; the mean was 0.92 mg/L (SD 0.17 mg/L).

Discussion

Determination of serum zinc by flameless atomic absorption spectrometry was first reported in 1973, by Kurz et al. (9). According to their procedure, readings from a 1-μL sample injected directly into the carbon rod atomizer were compared with values obtained from a standard curve prepared in demineralized water. Their evaluation of matrix effects was limited to single-point analytical-recovery experiments in serum, which gave values ranging from 89 to 108%. Although most of their samples were within the currently accepted reference interval for serum zinc, they made no direct comparison with another established method, as we have here.

Chooi et al. (10) reported significant matrix-related effects in measuring zinc in plasma by flameless atomic absorption spectrometry and concluded that it could not be used with either an aqueous standard curve or the method of additions. They used a plasma-based standard curve after correcting for a plasma blank, based on the assumption that a zinc-free plasma pool and plasma containing a large baseline amount of zinc would give curves of similar shape. From our data, we conclude that this assumption is not valid. They also noted a decrease in carbon cup sensitivity with the number of sequential firings of the furnace. We also observed this phenomenon initially. However, with new graphite tubes this particular effect disappeared, for unknown reasons. Whenever a new lot of carbon atomizer tubes or cups is acquired, the effect of sequential firings on sensitivity should be examined. If the effect is predictable, appropriate corrections can be made.

We evaluated matrix effects by comparing standard curve slopes in various solutions, as normalized to a water matrix slope, to eliminate day-to-day variations in instrument sen-
sitivity resulting from gas flows, component alignment, etc. We prepared an acceptable serum-like matrix that was relatively low in zinc by dialyzing either albumin (50 g/L), serum, or plasma (each containing 2 mmol of EDTA per liter to chelate the zinc and enhance its removal by dialysis) against a zinc-free synthetic solution of the principal inorganic constituents of serum. Correcting the standard curve for residual zinc in the added matrix provided an accurate reference for serum or plasma determinations and fully compensated for matrix effects. The matrix solution was dialyzed once every six to 12 months and stored frozen for use in preparing the standard curves for daily work. In the past, such interferences (which have been noted by several authors (11–14)) had been dealt with either by attempting to construct an artificial matrix (15) or by digestion of the sample. Sample digestion increases the risks of contamination and significantly prolongs sample processing. To avoid these problems, investigators have resorted to flame atomic absorption spectrometry techniques, which require larger samples.

The observed enhancement of sensitivity for various elements by HNO₃ (16–18) has been well known and frequently is used to advantage in flameless atomic absorption spectrometry. On the other hand, use of the dialysis-fluid solution as matrix markedly decreased the sensitivity, which was partly restored by the presence of albumin or serum proteins. Paradoxically, albumin in the absence of serum minerals also markedly decreased the sensitivity of the method. Apparently the interaction between serum proteins and minerals was such that the interference effects of both are decreased, resulting in increased sensitivity for zinc to 90% of the sensitivity in water.

Extending the reference curve into the nonlinear range improved the signal/noise ratio and yielded more satisfactory results. The “rational-method” calibration algorithm provided a simple, appropriate equation to compensate for the nonlinearity (7). This equation fits atomic absorption standard curves with as few as three parameters for a wide variety of analytes. Two parameters gave an excellent fit for zinc over the range of 0 to 30 μg/L (0 to 0.9 A) with 10 μg/L being near the top of the linear portion of the curve. To determine two parameters, only two standards were required; however, a random error in one of them would affect the interpolated results significantly. By deriving the least-squares solution for the parameters, multiple points on the standard curve can be used, with more-reliable results.

We validated the method by comparing results for unknown samples with those independently determined by another laboratory. The assay variance was greater than that for the flame method, owing to greater difficulty in handling small volumes and dilute solutions, but was still small enough for the technique to be clinically useful, and the advantage of small sample requirement may offset this disadvantage sufficiently to warrant its use. Furthermore, the standard de-

viation for normal serum zinc concentrations was less by this method than by flame atomic absorption. We also compared the normal zinc reference values in the present study with others reported in the literature (Table 2).

In summary, we have developed a precise micro-scale zinc determination for biological fluids, using 10 μL of sample and graphite furnace technology. Environmental zinc contamination was controlled by using appropriate disposable plastic ware, thoroughly de-ionized water, careful rinsing of pipet tips, and meticulous laboratory techniques. Matrix effects for urine determinations were variable, requiring that the method of additions be used. The matrix effects for serum or plasma were compensated for by adding albumin, serum, or plasma, each of which had been dialyzed to low zinc concentrations. These dialyzed preparations were added to the standard solution in the same concentration as in the samples. The serum sample concentrations were calculated from fitting a rational equation to the standard curve. These results agreed with independent determinations on the same samples by another laboratory, using established flame atomic absorption spectrometry methods.

We acknowledge the technical assistance of Cathy Zieba.

References


