

Use of a zinc fluorophore to measure labile pools of zinc in body fluids and cell-conditioned media

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BioTechniques 40:509-520 (April 2006)

Here we describe a rapid and sensitive zinquin-based fluorometric assay that enables one to monitor levels of labile Zn(II) in body fluids, buffers, and cell-conditioned culture media as well as changes in these pools in disease. Labile pools of Zn(II) are free or loosely bound pools and more tightly bound but zinquin-accessible pools in contrast to the fixed pools of Zn(II) within metalloproteins. In human plasma, mean labile Zn(II) was 8.1 μM (SEM 0.53; n = 81) and constituted about 70% of the total plasma Zn(II) and >90% of human plasma albumin Zn(II). Plasma labile Zn(II) was significantly depleted after 7 days of Zn(II) deprivation in mice, despite only small changes in body weight. Labile Zn(II) concentrations were also measured in the induced sputum plugs, saliva, and urine of normal adults and were 1.30 μM (SEM 0.27; n = 73), 0.11 μM (SEM 0.11; n = 6), and 0.23 μM (SEM 0.08; n = 8), respectively. Urinary labile Zn(II) concentration was significantly increased in some patients with type II diabetes mellitus (overall mean was 0.90 μM , SEM 0.30; n = 12). The technique may be particularly useful in assessing extracellular Zn(II) levels in diseases associated with altered Zn(II) homeostasis, identifying those subjects most in need of Zn(II) supplementation, and defining the optimum concentrations of available Zn(II) in buffers and culture media.

INTRODUCTION

One of the major advances in the understanding of the cellular biology of Zn(II) has been the development of techniques to visualize and quantify the dynamic intracellular pools of Zn(II) (1,2). Labile Zn(II) is bound weakly to proteins and other macromolecules as well as to low molecular weight thiols such as glutathione (3,4). Study of these chelatable or labile pools of Zn(II) has thrown new light on mechanisms underlying processes as diverse as cell secretion, apoptosis, neurotransmission, Ras-mediated signaling pathways, and zinc transport pathways (3–7). A relatively neglected area to date, however, has been the detection and measurement of labile Zn(II) in extracellular fluids, including the major fluids of the body and in vitro culture

secretions or other cell-conditioned media.

There are several reasons why it may be important to measure the concentration of labile extracellular Zn(II). It has been estimated that 23% of the world's population are Zn(II) deficient and at heightened risk of infection, growth retardation, and other manifestations of Zn(II) deficiency (8,9). Meta-analysis of trials conducted in Vietnam, Bangladesh, and Indonesia have found that dietary zinc supplementation reduces the incidence of pneumonia by 41% and diarrhea by 18% (9). Zn(II) deficiency also complicates a variety of chronic clinical conditions, such as sickle cell anemia, diabetes, rheumatoid arthritis, and asthma (10–12). Traditionally, serum or plasma Zn(II) concentrations, as measured by

spectrometry (AAS), have been used as markers of zinc deficiency. However, one limitation in using such measures is that a significant proportion of the total serum or plasma Zn(II) is tightly incorporated in the metalloprotein α -2-macroglobulin; the function of this Zn(II) is not known but it is poorly exchangeable and does not appear to be a determinant of Zn(II) nutrition (10,13). In fact, the bulk of the 2–3 g of Zn(II) in the body comprises structural and catalytic Zn(II) contained within several hundred Zn(II) metalloenzymes and other intra- and extracellular Zn(II) metalloproteins. These fixed pools of Zn(II) are largely unaffected, even in severe Zn(II) deficiency (3,10). For example, Zn(II) deprivation in rats resulted in a progressive fall in plasma Zn(II) levels to about 40% of initial levels but did not decline further despite prolonged deprivation (14).

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The most relevant plasma Zn(II) pool is thought to be the labile Zn(II) bound to albumin and en route for uptake by the various organ systems. Although the speciation of Zn(II) between different plasma pools has been estimated by fractionation studies coupled with a total Zn(II) measure such as by AAS, a laboratory test that would largely measure the labile Zn(II) content in extracellular fluids, against a background of fixed

Zn(II) in metalloproteins, would be an important adjunct in assessing Zn(II) deficiency.

There is also a need to measure labile Zn(II) in culture media because many cellular processes are affected by Zn(II) and a number of cytokines and growth factors contain, or are dependent upon, Zn(II) (10,15,16). The optimal levels of extracellular Zn(II) for in vitro cultures remain poorly defined. In addition, some cell

types release or actively secrete Zn(II) (17,18). A technique to measure labile Zn(II) in extracellular fluids would enable quantification of Zn(II) release from cells as well as provide a means to monitor available Zn(II) concentrations in a variety of buffers and media.

Our studies over the past decade have focused on the measurement and visualization of labile Zn(II) in subcellular compartments, whole cells, and

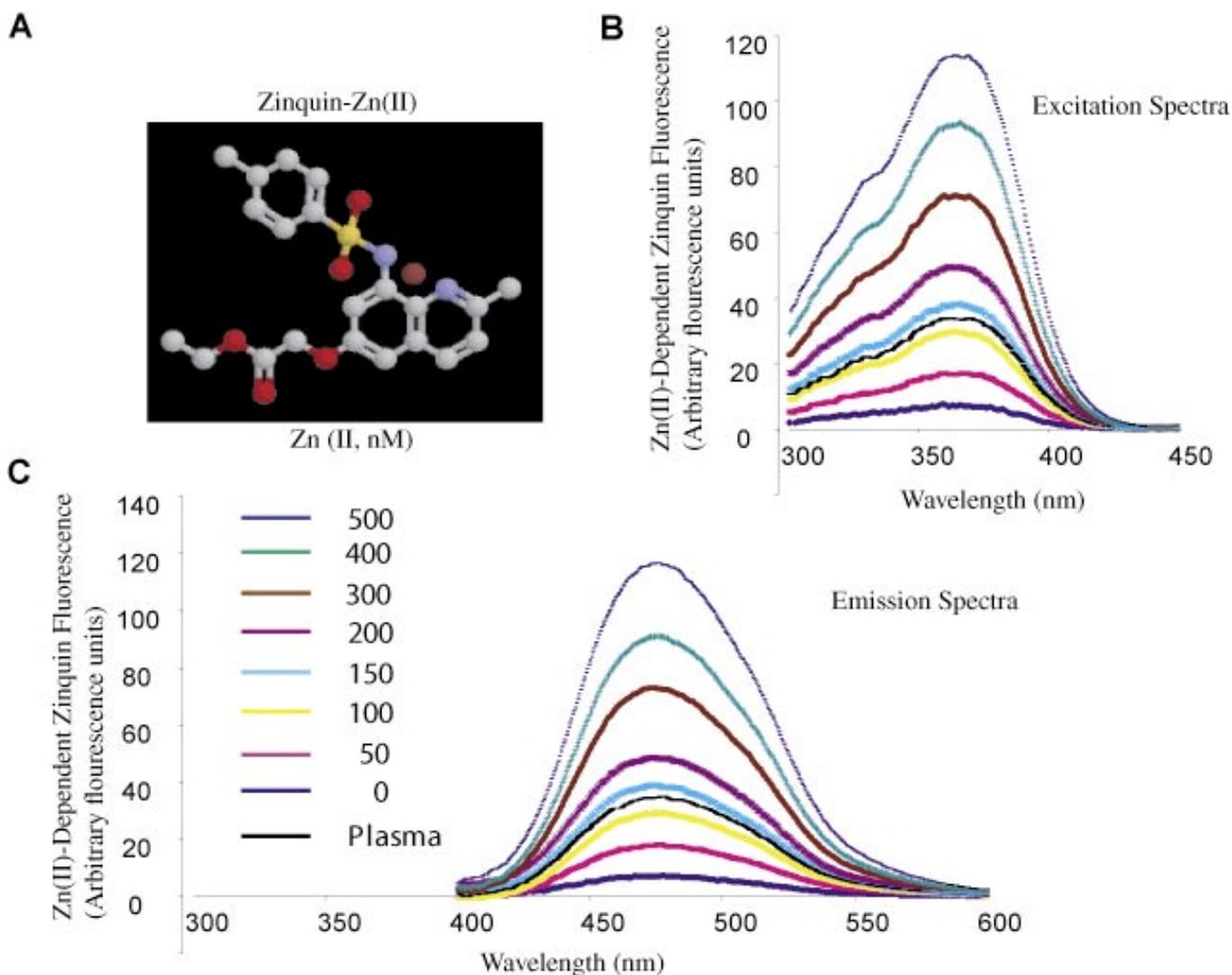


Figure 1. Structure of zinquin ethyl ester and its excitation and emission spectra with exogenous and endogenous Zn(II). (A) Structure of a 1:1 zinquin:Zn(II) complex. The Zn(II) ion is denoted by the magenta sphere between the two nitrogens (blue spheres), contributed by the quinoline ring and by the sulfonamido group. Red spheres indicate oxygen atoms, and the yellow sphere is sulfur. (B) Excitation spectra for zinquin (emission wavelength: 485 nm) in the presence of increasing final concentrations (0–500 nM, prepared using analytical grade zinc sulfate; Sigma-Aldrich) of Zn(II) in buffer A. Also shown is the excitation spectrum for fluorescence of zinquin with the endogenous Zn(II) in pooled human plasma (black line). Fluorescence in the presence of EGTA has been subtracted to give Zn(II)-dependent fluorescence. Note the major excitation peak at 365 nm and a shoulder at 340 nm; there was a negligible shift to higher wavelengths with increasing Zn(II). (C) Corresponding emission spectra for zinquin (excitation wavelength 365 nm). Note the single emission peak and the minimal shift to higher wavelengths with increasing Zn(II). Emission spectrum for fluorescence of zinquin with the endogenous Zn(II) in pooled human plasma is shown by the black line.

tissues using the UV-excitable fluorophore zinquin ethyl ester (Figure 1A) (12,18–20). Zinquin is sensitive to nM concentrations of free Zn(II), highly specific for Zn(II) among other metal cations, and preferentially reactive with the most labile (free or thermodynamically exchangeable) Zn(II) pools. Zinquin can also react with another category of Zn(II), which is Zn(II) partially complexed by a biomolecule such that binding sites remain available to zinquin (21). For this reason, the Zn(II) detected by zinquin includes both exchangeable Zn(II) and more tightly bound but accessible Zn(II). For the purposes of this manuscript, we use the term labile Zn(II) to include all zinquin-detectable Zn(II) that is capable of being quenched by the chelator EGTA. Here we have used zinquin in a rapid and sensitive fluorometric assay for the measurement of labile Zn(II) in minute volumes of plasma and other extracellular fluids.

MATERIALS AND METHODS

Materials

Zinquin [ethyl-(2-methyl-8-*p*-toluenesulfonamido-6-quinolyloxy)-acetate] was synthesized according to a previously published method (22). It can be obtained from Dr. David Ward (Department of Chemistry, University of Adelaide, Adelaide, South Australia; david.ward@adelaide.edu.au) or from several other commercial sources including Toronto Research Chemicals (North York, ON, Canada) and Sigma-Aldrich (St. Louis, MO, USA). Although these other preparations have not yet been tested in the labile plasma Zn(II) assay, they are identical in structure (or at least only differ in the nature of the ester group, which is unlikely to affect their performance in this assay). Each has been used successfully to image intracellular labile Zn(II) (23,24). All other reagents were reagent-grade, and water was Zn(II)-free (Milli-Q[®]; Millipore, Billerica, MA, USA).

Buffer A consisted of Zn-free Hank's balanced salt solution (HBSS) to which ovalbumin (OVA; Sigma-Aldrich) was added to a final concentration of 0.3 mg/

mL. To remove Zn(II), 5 g prewashed Chelex[®] 100 resin (1% sodium form; Sigma-Aldrich) were added to 50 mL HBSS. After rocking for 40 min at room temperature, the buffer was decanted and treated with a second cycle of Chelex 100. The pH after chelex treatment was 8.6 and was not readjusted because zinquin is optimal at alkaline pH. A similar procedure was used for the removal of Zn(II) from other fluids. OVA was added to buffer A because it is a Zn(II)-free protein and helps prevent the precipitation of zinquin from aqueous solutions.

Subjects and Samples

All procedures conformed to National Health and Medical Research guidelines and were approved by The Queen Elizabeth Hospital Human Ethics Committee (University of Adelaide, Woodville, Australia). Bloods were obtained from adults enrolled in The North West Adelaide Health Study and were collected into lithium heparin gel tubes. Plasma samples were obtained by centrifugation (1000× *g* for 10 min at 4°C) and stored at -20°C. Pooled human plasma was prepared from 50 individuals. Induced sputum (collected following inhalation of aerosolized saline) and saliva were taken from randomly chosen normal subjects. Mucin plugs were removed from sputum samples, and then the mucin plug was dissolved in 4 volumes of a 10% aqueous solution of Sputolysin[®] (Calbiochem, San Diego, CA, USA). After rocking for 15 min at room temperature, an equal volume of Zn(II)-free HBSS was added. Samples were filtered through nylon (60 μm pore size) and filtrates centrifuged (400× *g* for 10 min). Supernatants were removed and stored at -20°C. Urine was obtained from healthy control subjects and patients with type II diabetes mellitus attending the Endocrinology Clinic at The Queen Elizabeth Hospital. Creatinine and glucose concentrations were measured by standard techniques in the Department of Clinical Pathology, The Queen Elizabeth Hospital.

Bronchial Epithelial Cells and Cell Secretion Experiments

Human bronchial epithelial cells were brushed from the left and right main bronchus of adult subjects during routine bronchoscopy, after obtaining informed consent. All bronchoscopy procedures were performed at The Queen Elizabeth Hospital. The cells were collected into RPMI 1640 medium (Sigma-Aldrich) and placed on ice until further processing. To dissolve mucus, samples were diluted 1:5 with 10% Sputolysin, mixed for 15 min, and centrifuged (400× *g* for 3 min at room temperature). Cell pellets were washed and resuspended in bronchial epithelial cell growth medium (BEGM[®]): Dulbecco's modified Eagle's medium (DMEM), a 1:1 mixture of DMEM (Invitrogen, Grand Island, NY, USA) and BEGM with supplements (Cambrex BioScience, Walkerville, MD, USA). In some experiments, Zn(II)-depleted medium was used; this was replenished with Ca(II) and Mg(II) to 1 and 0.5 mM, respectively, and the pH was adjusted to 7.4 with 1 M HCl. Cultures were incubated for 3 h at 37°C in 5% CO₂ and 95% air. Culture supernatants were harvested by centrifugation (2 cycles of 12,000× *g* for 3 min) and stored at -20°C.

Measurement of Labile Zn(II) Concentrations in Fluids by Zinquin Fluorometry

Two fluorometric systems were tested and gave similar results. An LS50 Spectrofluorometer (Perkin Elmer, Wellesley, MA, USA) was used for initial experiments, including derivation of excitation and emission spectra and kinetic experiments. For the latter, a round 3-mL glass cuvette stirred by a magnetic stir bar was used. The tracing was allowed to stabilize for 50 s after each addition of reagents. Unless otherwise indicated, the excitation wavelength was 365 nm and the emission wavelength was 485 nm (5 nm slit widths). Other experiments were performed in a TBS-380 Mini Fluorometer (Turner BioSystems, Sunnyvale, CA, USA) with the instrument in UV fluorescence mode.

Both fluorometers used disposable 1-mL cuvettes (Halb-Mikro; Greiner Bio-One, Longwood, FL, USA). The TBS-380 also had an adapter for 100- μ L microcuvettes (Turner Designs, Sunnyvale, CA, USA), and these were used for measurements of Zn(II) in samples of low volume and/or low Zn(II) concentration. Twenty microliters of samples or standards were added to the cuvettes, and volumes were made up to 100 μ L with zinquin buffer A, pH 8.6 (Table 1). The lower detection limit was 10 pmol of Zn(II) in a 20- μ L volume [500 nM Zn(II) concentration in the biological sample].

Table 1 describes the method for measuring labile Zn(II) in plasma. Variations for other body fluids are given below. The following precautions need to be taken. Glassware must not be used because they are a ready source of contaminating Zn(II). All samples and buffers must be prepared in previ-

ously unused disposable plastic tubes. The usual precautions are required for minimizing Zn(II) contamination during collection and processing of body fluids. Microcentrifuge tubes can be used for samples and standards, and sterile 50-mL polystyrene centrifuge tubes (e.g., from Iwaki, Chiba, Japan) can be used for buffers; if a large volume of buffer is required, T-75 culture flasks can be used.

EGTA-containing cuvettes were used to obtain the nonlabile Zn-dependent fluorescence because the metal chelator EGTA eliminates any labile Zn-dependent zinquin fluorescence. Labile Zn(II) was derived by subtracting mean fluorescence in the presence of EGTA from that in the absence of EGTA and converting fluorescence into Zn(II) concentrations, using the linear portion of the standard curve.

The commercial preparations of Sputolysin, used for dissolving the mucin plugs in sputum plug samples, contained some contaminating Zn(II). To correct for this, on the day of sputum processing, an aliquot of the Sputolysin processing reagent (designated blank) was kept and stored with the samples at -20°C . Zn(II) standards were also prepared in the Sputolysin processing buffer (Sputolysin, final concentration 0.5% in $0.5\times$ HBSS). Fifty microliters of blanks or sputum supernatants were added to the cuvettes (three with EGTA and three without, as for the other body fluids). Specific fluorescence of blanks was subtracted from the specific fluorescence of the corresponding sputum supernatant(s), and the difference was converted to a concentration of Zn(II) using the standard curve. Generally, fluorescence of blanks was very low compared with those of sputum supernatants; however, if the Sputolysin was allowed to age for more than 2 weeks after opening the bottle, high blank fluorescences were often obtained. This is thought to be due to oxidation and release of more tightly bound Zn(II) from the dithiothreitol (DTT) in the Sputolysin. Fluorescence readings of sputum supernatant in the presence of EGTA were nearly always negligible. In all experiments with standard cuvettes, a constant final volume of 1 mL was used.

Total Zn(II) was measured by absorption at 214 nm in a PE 3030 Flame Atomic Absorption Spectrometer with a graphite furnace HGA 400 (Perkin Elmer) as previously described (11).

Sephadex® G25 Chromatography of Albumin, Zn(II)-Free Albumin, or Zn(II)-Supplemented Albumin

A solution of albumin (40 mg/mL in water) was prepared and split into three portions. One was Chelex 100-treated to prepare labile Zn(II)-free albumin; another portion was supplemented with Zn(II) by incubation with 125 μ M ZnSO_4 for 48 h at 4°C [to allow sufficient time for the Zn(II) to equilibrate with albumin]; some of the Chelex-treated albumin was also resupplemented with Zn(II) in the same way; the remaining portion of albumin was not treated further. These solutions (0.3 mL)

Table 1. Assay for Labile Zn(II) in Extracellular Fluids

<p>1. Prepare Samples</p> <p>Set up six cuvettes^a for each sample: three with EGTA and three without EGTA.</p> <p>Add 20 μL plasma to each cuvette^b.</p> <p>Add 20 μL 30 mM EGTA to the EGTA⁺ cuvettes and 20 μL Milli-Q-grade water to the EGTA⁻ cuvettes. Ensure that the EGTA mixes well with the sample.</p> <p>2. Prepare Standards</p> <p>To each of three cuvettes, add 20 μL ZnNO_3 (0, 1.25, 2.5, 5, 7.5, 10, 15, or 20 μM)^c.</p> <p>Add 20 μL Chelex-treated pooled plasma to cuvettes.</p> <p>3. Zinquin-Containing Buffer A</p> <p>Make up sufficient buffer A for the required number of cuvettes in 50-mL polystyrene centrifuge tubes or T-75 culture flasks if larger volumes are required.</p> <p>For each milliliter of buffer A required, add 0.1 mL Chelex-treated $10\times$ HBSS, 0.9 mL Milli-Q water, and 0.3 mg OVA.</p> <p>Immediately before addition of buffer A to the cuvettes, add 2 μL 5 mM zinquin stock (in DMSO) per milliliter of buffer A. Mix well and quickly pipet 960 μL buffer A into each cuvette.</p> <p>Incubate at least 40 min (no longer than 120 min) at room temperature in the dark.</p> <p>4. Fluorometry</p> <p>For spectrofluorometers, use excitation wavelength of 365 nm and emission wavelength of 485 nm; for filter fluorometers, use UV filter.</p> <p>For samples, subtract the mean fluorescence in EGTA⁺ cuvettes from the mean fluorescence in EGTA⁻ cuvettes. Convert the fluorescence difference into a labile Zn(II) concentration from the standard curve, allowing for any additional dilution factors made.</p>
<p>HBSS, Hank's buffered salt solution; OVA, ovalbumin; DMSO, dimethyl sulfoxide; AAS, atomic absorption spectrometry.</p> <p>^aDisposable 1-mL fluorometry-grade cuvettes. For very dilute samples or samples of low volume, use 100-μL microcuvettes with adaptor. Twenty microliter samples or standards are added to cuvettes and volumes are made up to 100 μL with buffer A.</p> <p>^bFor saliva, urine, and sputum, use 60, 50, and 50 μL, respectively. For sputums, Zn(II) concentrations need to be corrected for contaminating Zn(II) in Sputolysin.</p> <p>^cZnNO_3 (1 g/L; AAS-grade). For saliva and urine, replace Chelex-treated plasma with water, and for sputums, replace with Sputolysin-processing buffer.</p>

were diluted to 3 mL with Chelex-treated HBSS (pH 8.6) and applied to 10-mL Econo-Pac® 10 DG columns (Sephadex G25; Bio-Rad Laboratories, Hercules, CA, USA), previously equilibrated with the same buffer. After the 3 mL had entered the gel, it was washed through with 10 mL HBSS. Fractions of 500 µL were collected. Two hundred microliters of each were added to cuvettes, and the volume was made to 1 mL with HBSS containing 25 µM zinquin and 0.3 mg/mL OVA. The fluorescence of the blanks was subtracted to give Zn(II)-dependent fluorescence.

Murine Zn(II) Deficiency

All animal procedures conformed to National Health and Medical Research guidelines and were approved by The University of Adelaide and Queen Elizabeth Hospital Animal Ethics Committees. Young female Balb/c mice (eight per group) were housed in cages with raised steel mesh floors to limit

the mice eating their feces. They were then put on control [30 parts per million (ppm)] or Zn-poor (1 ppm) diets (ICN Biochemicals, Aurora, OH, USA). On day 7, the mice were weighed, anaesthetized, and terminally bled by cardiac puncture into heparinized tubes. Plasma was prepared and assayed for labile Zn(II).

Statistical Analyses

Differences between groups were analyzed by repeated measures or one-way analysis of variance (ANOVA) as required, with post-hoc Student Newman Keull test.

RESULTS

Spectra, Kinetics, and Concentration-Dependence Studies

Figure 1B shows the excitation spectra of zinquin (485 nm emission wavelength) with increasing concentrations (0–500 nM) of Zn(II) in buffer A. Fluorescence, in the presence of EGTA, has been subtracted. Fluorescence increased linearly with increasing Zn(II), but there was no shift in the peak excitation wavelength. Figure 1C shows the corresponding emission spectra (at excitation wavelength of 365 nm). There was a single emission peak with only a slight right-hand shift with increasing Zn(II) concentrations. The excitation and emission peaks for zinquin fluorescence in plasma (arrows in Figure 1, B and C) were similar to those for Zn(II) solutions in buffer A.

Figure 2A shows the rapid fluorescence response of zinquin to Zn(II) both in buffer A [final Zn(II) concentration of 700 nM; Figure 2A, upper panel] or in pooled human plasma (60 µL of plasma per cuvette; Figure 2A, lower panel). Fluorescence peaked within a few seconds and remained stable for

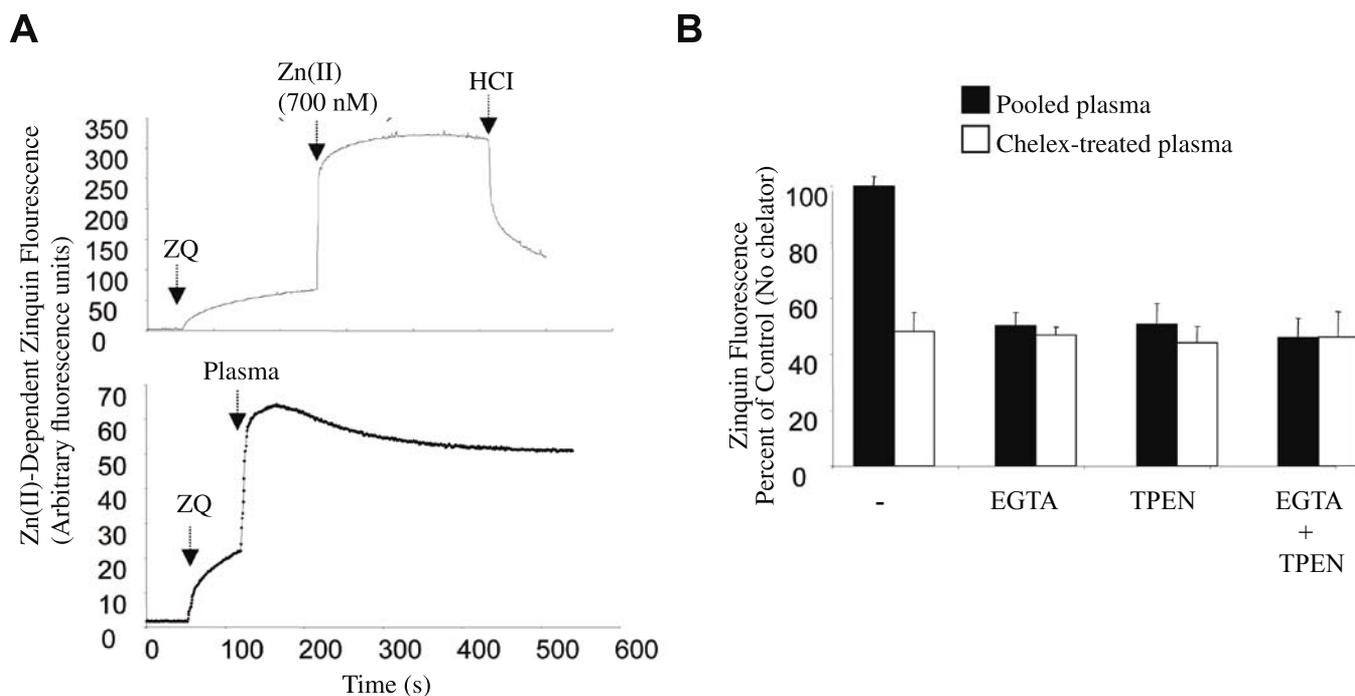


Figure 2. Kinetics of the zinquin-Zn(II) response and effect of Zn(II) chelators. (A) Rapid increases in Zn-dependent zinquin fluorescence after the addition of either 700 nM Zn(II) ions in buffer A (upper panel) or pooled human plasma (lower panel). In these initial experiments, zinquin was added to stirred cuvettes first. Buffers were not Chelex-treated, and the small increases in fluorescence with the pre-addition of zinquin were due to contaminating Zn(II) ions in the buffer. Zn-dependent fluorescence reached a stable level that was maintained for up to 60 min (data not shown). The addition of HCl (final concentration of 0.18%) caused rapid quenching of fluorescence. (B) Similar reductions in zinquin fluorescence of endogenous Zn(II) in pooled human plasma were achieved by pretreatment of plasma with Chelex 100 resin, EGTA, or N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), or combinations of these, suggesting that the reduction of fluorescence is due to a chelatable pool of Zn(II) in plasma and that the remaining fluorescence is chelator-insensitive. Data are the means of triplicates from a typical experiment. ZQ, zinquin.

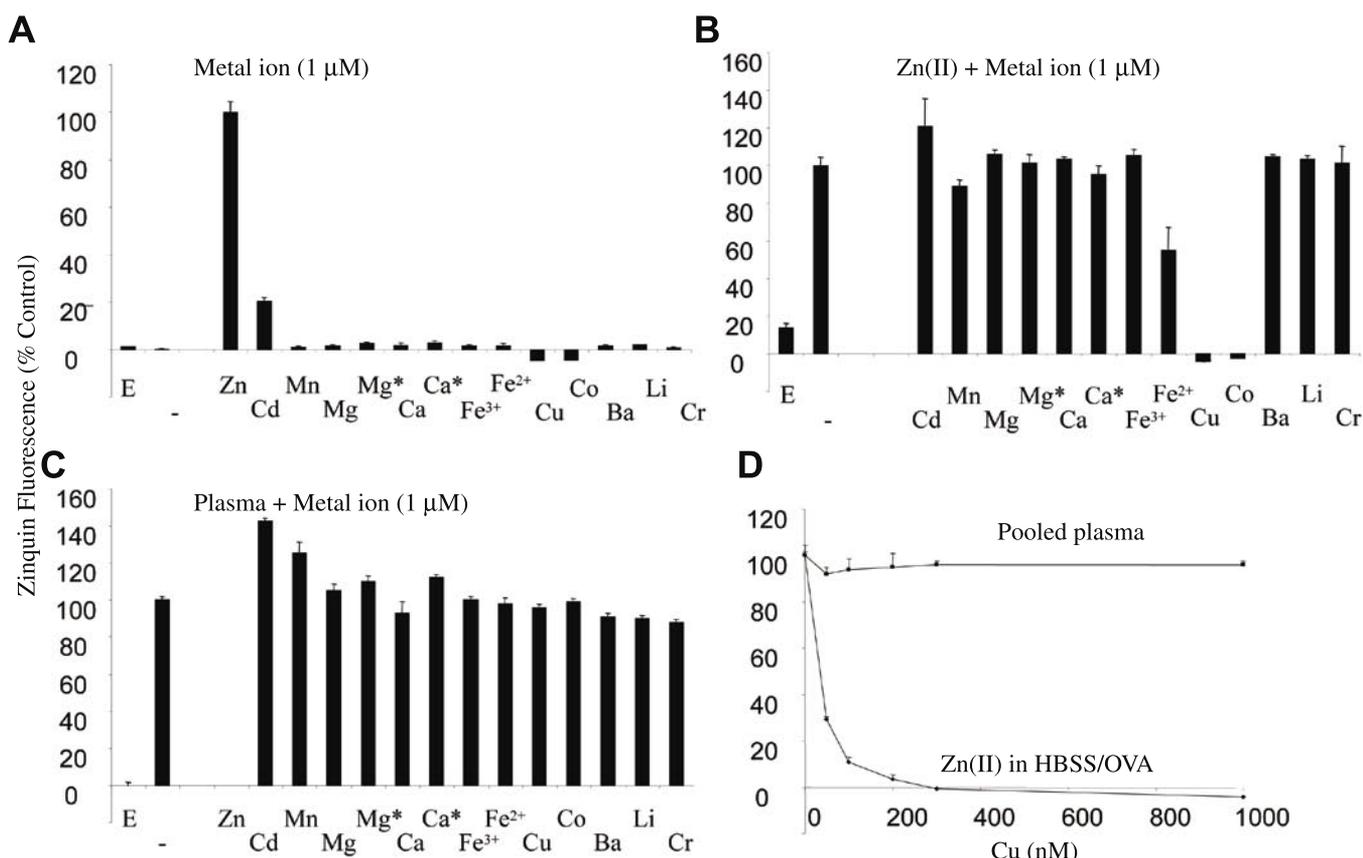


Figure 3. Metal ion specificity and interference. (A) Zinquin fluoresced weakly with Cd(II) and not at all with the other metal ions. All metal salts were at 1 μM (final concentration in cuvettes), except for Ca(II) and Mg(II), which were also tested at 50 and 40 μM , respectively (denoted by asterisks), to correspond to original plasma concentrations of 1.1 and 0.8 mM, respectively. Fluorescence of zinquin in the presence of Zn(II) was taken as 100% and fluorescence with other metals expressed relative to this. The first column “E” is the fluorescence of zinquin in the buffer with no added metal and in the presence of EGTA. The second column “-” is the fluorescence of zinquin in the buffer with no added metal ion. (B) The capacity of metal ions to interfere with Zn(II)-zinquin fluorescence in buffer A. All cuvettes contained 750 nM Zn(II) in addition to other metal ions (as in panel A). Fluorescence of zinquin in the presence of Zn(II) was taken as 100%, and this is indicated by the column “-”, where there was no competing metal ion present. As expected, EGTA quenched zinquin fluorescence with Zn(II). Cu(II) and Co(II) also completely quenched Zn(II)-dependent zinquin fluorescence. Cd(II) increased the fluorescence of zinquin, giving an apparent additive increase with Zn(II). (C) The capacity of metal ions to interfere with Zn(II)-zinquin fluorescence in the presence of plasma. Additions were the same as in panel A except that cuvettes also contained plasma [corresponding to about 750 nM total Zn(II)]. There was no quenching by Cu(II) and Co(II). Cd(II) showed additive effects with Zn(II) on fluorescence, and Mn(II) slightly increased zinquin fluorescence. Fluorescence of zinquin in plasma, with no other metal ion added, was taken as 100%. (D) Concentration-dependent quenching of zinquin fluorescence by Cu(II) in buffer but not in plasma. Same experimental conditions as in panel B except that cuvettes also contained increasing concentrations of Cu(II). Shown here is the complete quenching of Zn(II)-dependent fluorescence of zinquin by Cu(II) in buffer but no quenching when performed in the presence of plasma. Data are means of triplicates with standard deviations indicated by the error bars. HBSS, Hank’s balanced salt solution; OVA, ovalbumin.

up to 1 h. The addition of HCl (final concentration of 0.18%; Figure 2A, upper panel) caused a rapid decrease in fluorescence due to protonation of the nitrogens required for the binding of Zn(II).

Saturation plots of zinquin with Zn(II) in buffer A revealed two distinct phases of the fluorescence response (Supplementary Figure S1): at cuvette Zn(II) concentrations between 200 and 800 nM, there was a linear relationship between the Zn(II) concentration and zinquin fluorescence [2:1 zinquin:Zn(II) stoichiometry]; between 800 nM and 1.5 μM , the standard curve

began to plateau, and above 1.5 μM Zn(II), fluorescence increased rapidly again, reaching a second plateau at $>80 \mu\text{M}$ Zn(II) [1:1 zinquin:Zn(II) stoichiometry]. For measurement of Zn(II) in extracellular fluids, appropriate dilutions of the fluids were used to maintain cuvette Zn(II) concentrations ≤ 800 nM.

Effect of Zn(II) Chelators

Figure 2B shows the concentration-dependent quenching of zinquin fluorescence by the divalent cation chelator EGTA and the more specific

Zn(II) chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN). Zinquin fluorescence in pooled plasma was also decreased by these chelators but only partially, even at high concentrations of chelators. Prior removal of labile Zn(II) from plasma by two cycles of Chelex 100 resin treatment decreased subsequent zinquin fluorescence to the same extent as did pretreatment of the plasma with optimal concentrations of either TPEN or EGTA or with a combination of the two (Figure 2B). Furthermore, neither TPEN nor EGTA decreased zinquin fluorescence in Chelex-treated

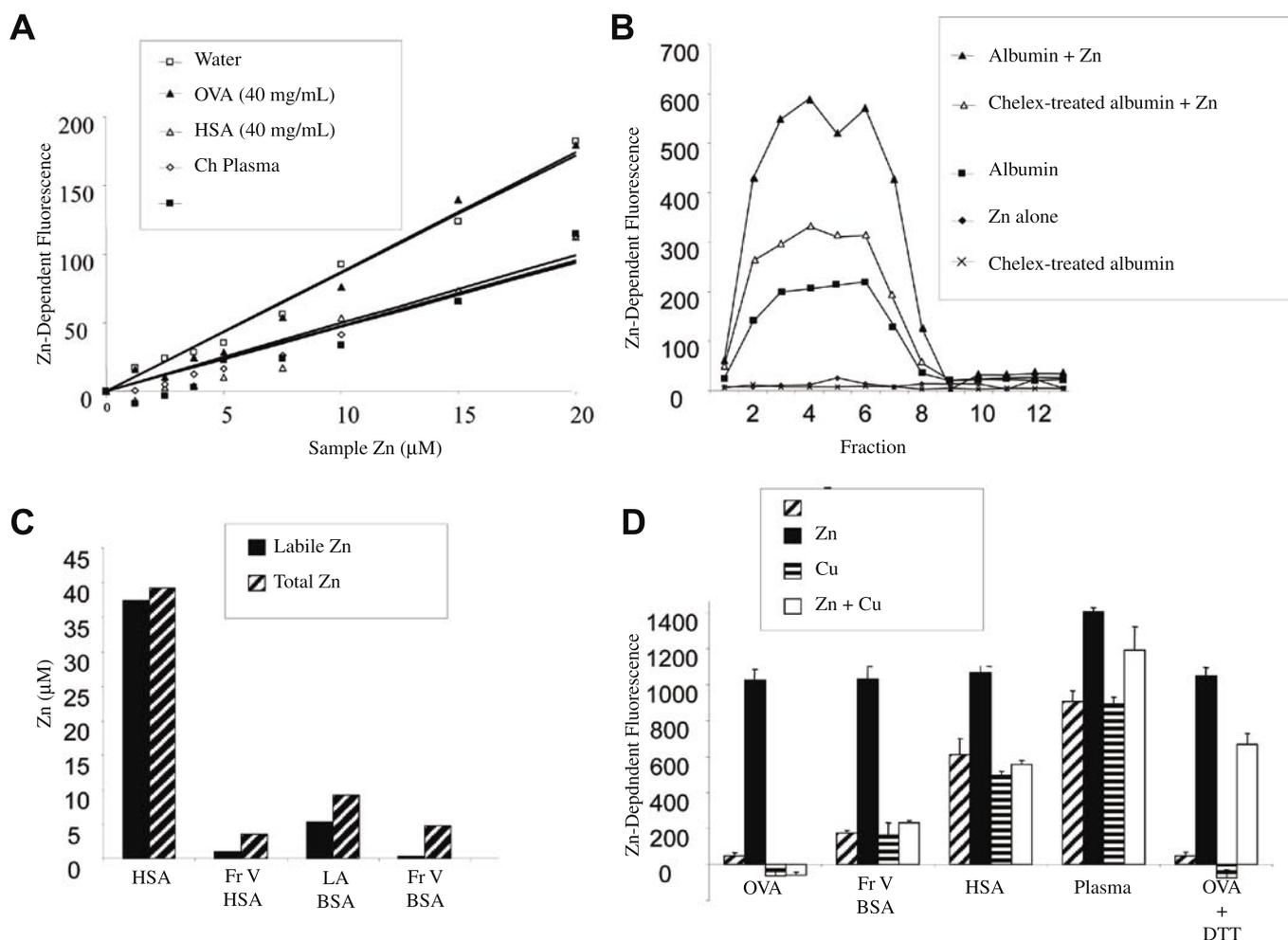


Figure 4. Zn(II) standards and labile Zn(II) in albumin. (A) Zn(II) standards [0–20 μM ; Zn(II) nitrate standard solution obtained from Merck (Whitehouse Station, NJ, USA)] were added to cuvettes to give final Zn(II) concentrations of 0–400 nM, in the presence of 20 μL of the following: water, ovalbumin (OVA; 40 mg/mL), nondenatured human serum albumin (40 mg/mL), Chelex-treated pooled human plasma (Ch plasma), or pooled human plasma. Shown is the reduction in the slope of the zinquin fluorescence standard curve for the albumin-containing samples, suggesting that some of the added Zn(II) interacts with albumin and becomes unavailable for zinquin. Fluorescence in the absence of added Zn(II) was subtracted. Data are means of triplicates for a typical experiment. (B) Zn-dependent fluorescence in albumin preparation was in the protein-containing void volume (first 7 \times 500 μL fractions) of Sephadex G25 desalting columns, indicating that the Zn(II) is complexed with albumin. Chelex-treated albumin lacked zinquin fluorescence while the addition of exogenous Zn(II) increased zinquin fluorescence. The fluorescence of the blanks was subtracted to give Zn-dependent fluorescence. (C) Labile and total Zn(II) in commercial albumin preparations (40 mg/mL). In particular, note the much higher Zn(II) content of the albumin prepared by the nondenaturing technique (Blue Sepharose chromatography). Linoleic acid-albumin (Sigma-Aldrich) had a moderate amount of Zn. Albumins prepared by Cohn fractionation (human Cohn fraction V, BSA Cohn fraction V; Sigma-Aldrich) had only small amounts of Zn(II) detectable by zinquin or AAS. (D) Cu(II) strongly quenched zinquin fluorescence in buffer A containing exogenous Zn(II) ions (first set of columns). Small volumes of plasma in the cuvette prevented Cu(II) quenching (fourth set of columns). Nondenatured human albumin significantly suppressed quenching, suggesting that plasma albumin may be the protective factor. Cohn fraction V BSA was much less effective. A low molecular weight thiol reagent, dithiothreitol, also prevented quenching. HSA, human serum albumin; BSA, bovine serum albumin; DTT, dithiothreitol; AAS, atomic absorption spectrometry.

plasma. Therefore, labile plasma Zn(II) as defined by the EGTA- or TPEN-sensitive portion of zinquin fluorescence is equivalent to the pool of Zn(II) removed by Chelex 100 treatment. By flame AAS, total Zn(II) content of plasma was reduced from 11.2 to 6.4 μM by Chelex 100 treatment, suggesting that approximately 40% of plasma Zn(II) is in the labile chelatable pool. By comparison, approximately

50% of zinquin fluorescence in plasma was chelator-sensitive.

Metal Specificity of Zinquin Fluorescence and Interference by Other Metals

Figure 3A shows the lack of capacity of various metal ions (1 μM) to induce fluorescence in zinquin in buffer A. The only metal ion other than Zn(II)

to give a significant (although weak) fluorescence with zinquin was Cd(II). To investigate metal ion interference, the experiment was repeated, but this time with all cuvettes containing 750 nM Zn(II) in addition to other metal ions (Figure 3B). Cd(II) increased fluorescence in an additive manner with Zn(II), whereas there was significant quenching of zinquin-Zn(II) fluorescence by Fe(II) and very strong

quenching by Cu(II) and Co(II). Cu(II) was effective even when Zn(II) was in a 15-fold molar excess. However, no quenching was observed when cuvettes contained at least 20 μL of pooled human plasma (Figure 3, C and D).

Titration Studies

To determine whether there was a linear relationship between zinquin fluorescence and plasma labile Zn(II) concentration, the following experiment was performed. Pooled plasma was depleted of labile Zn(II) by Chelex 100 treatment. Samples were then spiked with varying concentrations of Zn(II) from 2.5 to 30 μM , and varying volumes (0–40 μL) of these spiked plasmas were added to cuvettes. Fluorescence of unsupplemented Chelex-treated plasma was subtracted from the other data. Zinquin fluorescence increased linearly with the volume of plasma for all except the highest (30 μM) spiked zinc concentrations (Supplementary Figure S2). The latter reached a plateau beyond 30 μL volume due to saturation of zinquin by Zn(II). The regression line for Chelex plasma plus 20 μM Zn(II) was close to that for HBSS plus 20 μM Zn(II).

We then determined whether the slope of the Zn(II) standard curve was influenced by protein or serum in the assay buffer. Zn(II) standards (0–20 μM in Milli-Q water) were added to cuvettes to give final Zn(II) concentrations of 0–400 nM. To different sets of standards were added 20 μL of one of the following: water, OVA (40 mg/mL), human serum albumin (HSA, 40 mg/mL), pooled human plasma, or Chelex-treated pooled human plasma. The volume was then made to 1 mL by the addition of HBSS containing 10 μM zinquin and 0.3 mg/mL OVA. Fluorescence in the absence of added Zn(II) was subtracted from other values for each set. Figure 4A shows that the slopes of the regression lines for zinquin fluorescence of Zn(II) in the absence of exogenous protein (water) and in the presence of 40 mg/mL OVA were similar (8.7 and 8.6, respectively). The slopes of the regression lines for zinquin fluorescence of Zn(II) in the presence of human plasma, Chelex-treated plasma, or purified HSA were

substantially lower (4.7, 5.0, and 4.8, respectively; Figure 4A).

Labile Zn(II) in Albumin

Zinquin fluoresced strongly when added to a commercial preparation of purified nondenatured HSA (40 mg/mL, a typical plasma concentration). Most of the fluorescence was quenched by EGTA, suggesting that it was due to labile Zn(II). In order to ensure that zinquin was reacting with Zn(II) bound to albumin and not to contaminating free Zn(II) in the preparation, HSA was first applied to a Sephadex G25 desalting column and washed through with Zn(II)-free HBSS. Specific Zn(II)-dependent zinquin fluorescence was found in the albumin-containing void volume (fractions 1–7, albumin trace in Figure 4B). Free Zn(II) binds to the resin (25) and therefore appears neither in the void volume nor the subsequent wash-through [Zn(II) alone trace in Figure 4B]. When albumin was pretreated with 2 cycles of Chelex 100 to remove labile Zn(II), there was no zinquin fluorescence in the void volume (bottom tracing in Figure 4B), confirming that zinquin is reacting with a chelatable pool of Zn(II) in albumin. Treatment of Chelex-treated albumin with exogenous Zn(II) restored zinquin fluorescence in the albumin-containing void volume, and treatment of intact albumin with exogenous Zn(II) further increased zinquin fluorescence in the albumin fractions (upper two tracings, Figure 4B).

Figure 4C (first set of columns) shows that this solution of HSA contained 39.2 μM Zn(II) by AAS (hatched column), corresponding to an approximate molar ratio of 1 Zn(II) to 11.5 albumins. Ninety-two percent of this Zn(II) was labile Zn(II) as detected by zinquin (Figure 4C, filled column). In a commercial preparation of linoleic acid (LA)-bovine serum albumin (BSA) prepared by a nondenaturing technique, 56% of the Zn(II) was labile (Figure 4C, third set of columns). By contrast, albumins (human or bovine) prepared by the denaturing Cohn fractionation method contained much less total Zn(II) and relatively little of this was labile (Figure 4C, second and fourth sets of columns), suggesting that

Cohn fractionation removes the major pool of labile zinquin-reactive Zn(II) in albumin.

Albumin and Thiol Groups Prevent Cu-Mediated Quenching of Zinquin-Zn(II) Fluorescence

Figure 4D shows that albumin is also likely to be the factor in plasma that prevents Cu(II) from quenching Zn(II)-dependent zinquin fluorescence. When cuvettes contained human nondenatured albumin instead of plasma (third set of columns), there was significant reduction of Cu(II) quenching. However, Cohn-fractionated BSA was only weakly protective, at the same protein concentration. Albumin may prevent Cu(II) quenching by preferentially chelating this metal ion via its cysteine sulfhydryl groups. If this were true, the addition of excess thiol reagent to the cuvette should have a similar protective effect. This is shown in the final set of columns in Figure 4D where, in the presence of DTT, Cu(II) quenching was greatly reduced.

Depletion of Labile Plasma Zn(II) in Murine Zn(II) Deficiency

Figure 5A shows that labile plasma Zn(II) is significantly decreased in mice maintained for 7 days on a Zn-poor diet. There was only a modest decrease in body weight for the Zn(II)-deficient mice, falling from 18.5 ± 0.5 g in the Zn(II)-replete group to 16.9 ± 0.2 g ($P < 0.05$). Plasma labile Zn(II) concentration decreased by 39% ($P < 0.005$) in the Zn(II)-deficient group compared with the replete group, falling from 4.7 ± 0.5 μM to 2.9 ± 0.3 μM (Figure 5A).

Labile Zn(II) in Human Body Fluids and Cellular Secretions

As can be seen from Figure 5B, there was a wide variation in plasma labile Zn(II) between normal, randomly chosen adults ($n = 81$). The mean labile Zn(II) concentration was 8.1 μM (SEM, 0.53), constituting 72% of total plasma Zn(II) as measured by AAS (mean, 11.2 μM ; SEM, 0.22).

Zinquin was also used to measure labile Zn(II) in urine. Unlike the case

with plasma, Cu(II) strongly quenched zinquin fluorescence in urine. For a group of eight healthy adult subjects, mean urinary labile Zn(II) was 0.23 μM (SEM, 0.08). Labile urinary Zn(II) constituted 20% of total urinary Zn(II) as measured by AAS, and the Pearson correlation coefficient between the two was 0.65 (significant at 0.01 level, two-tailed). As shown in Figure 5C, mean urinary labile Zn(II) was significantly increased in a group of 12 patients with type II diabetes mellitus (range, 0.06–3.2 μM ; mean, 0.90; SEM, 0.3; $P < 0.005$). In a larger series comprising 23 patients with type II diabetes, 10 of these (44%) had labile urinary Zn(II) concentrations at least two standard

deviations greater than the mean values for healthy subjects. There was a reasonable correlation, trending to significance, between labile Zn(II)/creatinine ratio in urine and glucose/creatinine ratio ($n = 7$; Pearson correlation coefficient of 0.61; $P = 0.09$); larger numbers are required to substantiate this correlation.

The mean labile sputum plug Zn(II) concentration in 73 normal subjects was 1.30 μM (SEM, 0.27) compared with a mean total sputum plug Zn(II) concentration of 1.4 μM ($n = 66$; SEM, 0.63). This indicates that most of the Zn(II) in sputum plugs was labile Zn(II). There was a significant correlation (Pearson correlation coefficient

of 0.587) between total and labile Zn(II) in induced sputum ($P < 0.01$, two-tailed test). Salivary labile Zn(II) was much lower, with a mean of 0.11 μM (SEM, 0.11; $n = 6$).

Zinquin was also used to measure Zn(II) in culture medium used for growing primary bronchial epithelial cells. This medium contained 1.1 μM labile Zn(II) (SEM, 0.01). This was decreased to $0.1 \pm 0.01 \mu\text{M}$ Zn(II) ions by two cycles of Chelex 100 treatment (Figure 5D). When normal primary human bronchial epithelial cells were cultured in the Zn(II)-depleted medium for 3 h, the cells released 0.7 μmol Zn(II)/750- μL well volume (basal secretion), restoring the labile Zn(II)

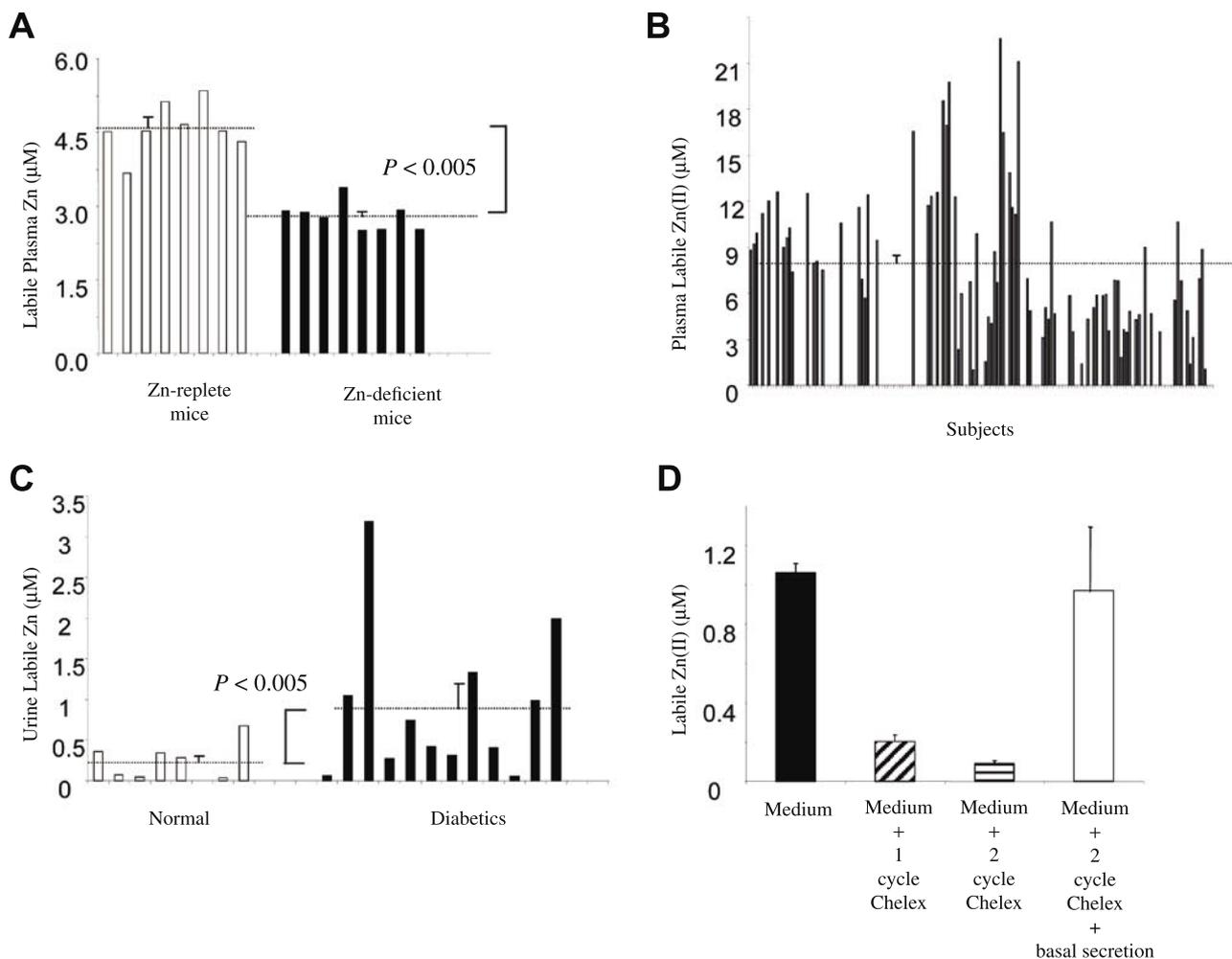


Figure 5. Labile Zn(II) in murine Zn(II) deficiency, human physiological fluids, and cell secretions. (A) Shown is significantly decreased plasma labile Zn(II) in Balb/c mice fed a Zn-poor diet (1 ppm) Zn(II) for 7 days compared with Zn-replete mice maintained on a normal diet (30 ppm Zn). There were eight mice per group; dotted lines show the mean values, and error bars show standard errors of means (SEM). (B) The figure shows the distribution of plasma labile Zn(II) in 81 randomly chosen human subjects. Dotted lines show the mean values, and error bars show the SEM. (C) The figure shows increased urinary labile Zn(II) in most patients with type II diabetes mellitus compared with healthy subjects. Dotted lines show the mean values, and error bars show the SEM. (D) Shown is the removal of labile Zn(II) from culture medium by two cycles of Chelex 100 treatment and the release of labile Zn(II) into the medium by bronchial epithelial cells. ppm, parts per million.

concentration in the culture medium to approximately 1 μM .

DISCUSSION

This study reports a sensitive, reliable, and simple method for monitoring labile Zn(II) levels in extracellular fluids. The method was optimized with respect to the kinetics, volumes of samples, and fluorophore concentration. In plasma, it largely detects albumin Zn(II). Because most studies have concluded that the main exchangeable transport pool of Zn(II) resides in the albumin fraction and this protein is involved in cellular Zn(II) uptake (10,13,14,26–29), it is clearly important to have a selective measure of the labile, albumin-bound pools. Estimates of albumin Zn(II) range from 30% to 85% of total plasma Zn(II) (26,27,30). Some of this variation is likely due to the separation techniques used. While Blue Sepharose chromatography does not displace Zn(II) from serum albumin (27), Cohn fractionation of plasma resulted in a loss of labile Zn(II) from albumin. Chelex 100 resin has been found to specifically deplete the albumin-Zn(II) from plasma, leaving behind the α -2-macroglobulin-Zn(II) (31). In support of this, our studies have shown that the chelator-sensitive component of zinquin fluorescence in plasma was depleted by Chelex 100 treatment while zinquin reacted strongly with Zn(II) in purified nondenatured albumin. Zinquin may therefore prove to be a selective marker of albumin Zn(II), obviating the need to separate albumin from other plasma proteins prior to the analysis of Zn(II).

Not all albumin Zn(II) was accessible to and/or reactive with zinquin. This was clear from the results of two different types of experiments. First, when Zn(II) was added to cuvettes in the presence of HSA, a portion of the added Zn(II) was no longer detectable by zinquin (Figure 4A); similar results were obtained with the addition of Zn(II) in the presence of human plasma, either Chelex-treated or untreated. Second, analysis of the endogenous Zn(II) in Cohn-fractionated albumin showed that zinquin fluoresced with only a minor portion of the total Zn(II) detectable by AAS (Figure 4D). Therefore, for the analysis of Zn(II) in serum/plasma or other body fluids, Zn(II)

standards must be matched to the protein matrix of the samples. For this purpose, Chelex-treated pooled plasma is ideal.

Specificity of the zinquin fluorometric assay for Zn(II) was confirmed by showing that the only other metal ion capable of inducing zinquin fluorescence was Cd(II). This is unlikely to be important in most situations because the signal was weak [about 20% of that given by an equimolar concentration of Zn(II)] and there is a large excess of Zn(II) over Cd(II) in the body (10). Of more importance is the potential for Cu(II) ions to quench Zn(II)-induced zinquin fluorescence. However, while this quenching occurred in simple buffers, there was no quenching by Cu(II) in the presence of plasma. The most likely explanation for this is mopping up of exogenous Cu(II) by plasma proteins, not only by ceruloplasmin, a major Cu transport protein in plasma (32), but also albumin, which has a strong Cu binding site that is four orders of magnitude stronger than that of Zn(II) to albumin (33). The inability of OVA [which lacks Cu(II)-reactive cysteine thiol residues] to prevent quenching and the strong protection against Cu(II) quenching by DTT suggests that plasma albumin sulfhydryls are required.

The findings in mice, that labile Zn(II) was significantly depleted within 7 days of consuming a Zn-free diet, and in the human population study, that about 15% of subjects had labile Zn(II) levels more than two standard deviations below the mean, suggests that measures of labile plasma Zn(II) may help to identify subjects at risk of Zn(II) deficiency. Plasma Zn(II), however, may not adequately represent tissue Zn(II) concentrations and therefore it may be more appropriate to measure labile Zn(II) in extracellular fluids within these tissues. Low but detectable concentrations of labile Zn(II) were detected in other body fluids, including induced sputum plugs. In studies to be reported separately, we have shown that labile Zn(II) is significantly decreased in the fluid phase of the induced sputum plugs of patients with asthma (manuscript in preparation). Finally, the use of zinquin to measure labile Zn(II) in urine may provide a rapid assay for

measuring renal losses of Zn(II). Our study has confirmed the previously reported hyperzincuria in type II diabetes mellitus (34). In particular, measurement of urinary labile Zn(II) identified a subpopulation of diabetic patients with altered Zn(II) homeostasis. These results suggest that measurement of labile Zn(II) in plasma and other body fluids will be useful in rapidly assessing Zn(II) status in disease. It should also provide a convenient on-site test for body fluid Zn(II) that can be used during Zn(II) supplementation trials both to monitor the effectiveness of the supplementation and to identify those most in need of further Zn(II).

The zinquin assay can also be used as a surrogate measure of plasma Zn(II) in situations where AAS is unavailable or where plasma volumes or concentrations are too low for reliable AAS measurements. In addition to a relatively inexpensive filter fluorometer (approximately US \$7000), the fluorometric assay uses 16 μg of zinquin (approximately 70 cents) per plasma sample. Although the consumable costs per sample by AAS are very low, the major expense is in the purchase of an instrument. A typical charge for the measurement of Zn(II) by a trace elements laboratory is US \$20 per sample.

In conclusion, this manuscript describes a rapid, sensitive, and specific fluorometric technique for the measurement of labile Zn(II) pools in microliter volumes of plasma, other body fluids, and culture supernatants. The technique may be particularly useful in assessing extracellular Zn(II) levels in primary and secondary Zn(II) deficiencies and defining concentrations of available Zn(II) in culture media. However, it should be noted that zinquin also has the potential to react with those pools of protein-associated Zn(II) that, despite being more tightly bound to protein, retain one or more ligand binding sites for zinquin (21).

ACKNOWLEDGMENTS

We would like to acknowledge the technical assistance provided by Cassie Hilditch, Anita Cerutti, Jane Engler, and Amy Zanker. We are also grateful

to Sandy Pickering and Dr. George Philipov for assistance with obtaining the patient samples, Dr. Peter Barreau for the AAS measurements, and Drs. Indu Mahadevan and Marc Kimber for the synthesis of zinquin. Funding was provided by the National Health and Medical Research Council and grants from the University of Adelaide and The Queen Elizabeth Hospital Research Foundation.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

- Danscher, G. 1996. The autometallographic zinc-sulphide method. A new approach involving in vivo creation of nanometer-sized zinc sulphide crystal lattices in zinc-enriched synaptic and secretory vesicles. *Histochem. J.* 28:361-373.
- Frederickson, C.J. 2003. Imaging zinc: old and new tools. *Sci. STKE.* 182:pe18.
- Frederickson, C.J. 1989. Neurobiology of zinc and zinc-containing neurons. *Int. Rev. Neurobiol.* 31:145-238.
- Truong-Tran, A.Q., R.E. Ruffin, and P.D. Zalewski. 2001. The role of zinc in caspase activation and apoptotic cell death. *Biometals* 14:315-330.
- Frederickson, C.J., J.Y. Koh, and A.I. Bush. 2005. The neurobiology of zinc in health and disease. *Nat. Rev. Neurosci.* 6:449-462.
- Palmiter, R.D., T.B. Cole, and S.D. Findley. 1996. ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. *EMBO J.* 15:1784-1791.
- Bruinsma, J.J., T. Jirakulaporn, A.J. Muslin, and K. Kornfeld. 2002. Zinc ions and cation diffusion facilitator proteins regulate Ras-mediated signaling. *Dev. Cell* 2:567-578.
- Sandstead, H.H. 1991. Zn(II) deficiency. A public health problem. *Am. J. Dis. Child.* 145:853-859.
- Walker, C.F. and R.E. Black. 2004. Zinc and the risk for infectious disease. *Annu. Rev. Nutr.* 24:255-275.
- Vallee, B.L. and K.H. Falchuk. 1993. The biochemical basis of zinc physiology. *Physiol. Rev.* 73:79-118.
- Truong-Tran, A.Q., R.E. Ruffin, P.S. Foster, A.M. Koskinen, P. Coyle, J.C. Philcox, A.M. Rofe, and P.D. Zalewski. 2002. Altered zinc homeostasis and caspase-3 activity in murine allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 27:286-296.
- Zalewski, P.D., A.Q. Truong-Tran, D. Grosse, L. Jayaram, C. Murgia, and R.E. Ruffin. 2005. Zinc metabolism in airway epithelium and airway inflammation: basic mechanisms and clinical targets. *Pharmacol. Ther.* 105:127-149.
- Smith, K.T., M.L. Failla, and R.J. Cousins. 1979. Identification of albumin as the plasma carrier for zinc absorption by perfused rat intestine. *Biochem. J.* 184:627-633.
- Wilkins, P.J., P.C. Grey, and I.E. Dreosti. 1972. Plasma zinc as an indicator of zinc status in rats. *Br. J. Nutr.* 27:113-120.
- Ibs, K.H. and L. Rink. 2003. Zinc-altered immune function. *J. Nutr.* 133(Suppl 1):1452S-1456S.
- Zalewski, P.D. 1996. Zinc and immunity: implications for growth, survival and function of lymphoid cells. *J. Nutr. Immunol.* 4:39-101.
- Qian, W.J., J.L. Peters, G.M. Dahlgren, K.R. Gee, and R.T. Kennedy. 2004. Simultaneous monitoring of Zn²⁺ secretion and intracellular Ca²⁺ from islets and islet cells by fluorescence microscopy. *BioTechniques* 37:922-930.
- Ho, L.H., R.E. Ruffin, C. Murgia, L. Li, S.A. Krilis, and P.D. Zalewski. 2004. Labile zinc and zinc transporter ZnT₄ in mast cell granules: role in regulation of caspase activation and NF-κB translocation. *J. Immunol.* 172:7750-7760.
- Ward, A.D., S.F. Lincoln, W.H. Betts, P.D. Zalewski, I.J. Forbes, I. Mahadevan, M. Kimber, and K. Hendrickson. 2000. Zinquin ester—a reagent for the investigation of the role of available Zn(II) in living systems, p. 995-998. *In* A.M. Rousell, R.A. Anderson, and A.E. Favier (Eds.), *Trace Elements in Man and Animals*. Kluwer Academic, New York.
- Coyle, P., P.D. Zalewski, J.C. Philcox, I.J. Forbes, A.D. Ward, S.F. Lincoln, I. Mahadevan, and A.M. Rofe. 1994. Measurement of zinc in hepatocytes using a fluorescent probe, zinquin: relationship to metallothionein and intracellular zinc. *Biochem. J.* 303:781-786.
- Hendrickson, K.M., J.P. Geue, O. Wyness, S.F. Lincoln, and A.D. Ward. 2003. Coordination and fluorescence of the intracellular Zn²⁺ probe [2-methyl-8-(4-toluenesulfonamido)-6-quinolyloxy]acetic acid (Zinquin A) in ternary Zn²⁺ complexes. *J. Am. Chem. Soc.* 125:3889-3895.
- Mahadevan, I.B., M.C. Kimber, S.F. Lincoln, E.R.T. Tiekink, A.D. Ward, W.H. Betts, I.J. Forbes, and P.D. Zalewski. 1996. The synthesis of zinquin ester and zinquin acid, zinc (II)-specific fluorescing agents for use in the study of biological zinc (II). *Aust. J. Chem.* 49:561-568.
- Tang, Z.L., K. Wasserloos, C.M. St. Croix, and B.R. Pitt. 2001. Role of zinc in pulmonary endothelial cell response to oxidative stress. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 281:L243-L249.
- Tsuda, M., K. Imaizumi, T. Katayama, K. Kitagawa, A. Wanaka, M. Tohyama, and T. Takagi. 1997. Expression of zinc transporter gene, ZnT-1, is induced after transient forebrain ischemia in the gerbil. *J. Neurosci.* 17:6678-6684.
- Lonnerdal, B., B. Hoffman, and L.S. Hurley. 1982. Zinc and copper binding proteins in human milk. *Am. J. Clin. Nutr.* 36:1170-1176.
- Faure, H., A. Favier, M. Tripier, and J. Arnaud. 1990. Determination of the major zinc fractions in human serum by ultrafiltration. *Biol. Trace Elem. Res.* 24:25-37.
- Foote, J.W. and H.T. Delves. 1984. Albumin bound and alpha 2-macroglobulin bound zinc concentrations in the sera of healthy adults. *J. Clin. Pathol.* 37:1050-1054.
- Rowe, D.J. and D.J. Bobilya. 2000. Albumin facilitates zinc acquisition by endothelial cells. *Proc. Soc. Exp. Biol. Med.* 224:178-186.
- Stewart, A.J., C.A. Blindauer, S. Berezhenko, D. Sleep, and P.J. Sadler. 2003. Interdomain zinc site on human albumin. *Proc. Natl. Acad. Sci. USA* 100:3701-3706.
- Chesters, J.K. and M. Will. 1981. Measurement of zinc flux through plasma in normal and endotoxin-stressed pigs and the effects of zinc supplementation during stress. *Br. J. Nutr.* 46:119-130.
- Inagaki, K., N. Mikuriya, S. Morita, H. Haraguchi, Y. Nakahara, M. Hattori, T. Kinoshita, and H. Saito. 2000. Speciation of protein-binding zinc and copper in human blood serum by chelating resin pre-treatment and inductively coupled plasma mass spectrometry. *Analyst* 125:197-203.
- Frieden, E. 1986. Perspectives on copper biochemistry. *Clin. Physiol. Biochem.* 4:11-19.
- Masuoka, J., J. Hegenaue, B.R. Van Dyke, and P. Saltman. 1993. Intrinsic stoichiometric equilibrium constants for the binding of zinc(II) and copper(II) to the high affinity site of serum albumin. *J. Biol. Chem.* 268:21533-21537.
- Kinlaw, W.B., A.S. Levine, J.E. Morley, S.E. Silvis, and C.J. McClain. 1983. Abnormal zinc metabolism in type II diabetes mellitus. *Am. J. Med.* 75:273-277.

Received 25 October 2005; accepted 22 December 2005.

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