Measurement of Labile Pools of Zinc in Body Fluids and Cell-Conditioned Media using the Turner Biosystems TB380 Fluorometer

This application note was submitted to Turner BioSystems under the "Tell us your story program" by Dr. Peter Zalewski of University of Adelaide

1. INTRODUCTION

A technique is described here to measure free or loosely-bound (labile, exchangeable) Zn(II) in extracellular fluids, including the major fluids of the body and *in vitro* culture secretions or other cell-conditioned media (for full description see Reference 1). There are several reasons why it may be important to measure the concentration of labile extracellular Zn(II). 1) Traditionally, serum or plasma Zn(II) concentrations, as measured by processes such as atomic absorption 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 a-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) nutriture. 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. 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. 2) The assay for labile Zn(II) can be used as a surrogate measure of body fluid Zn(II) in situations where AAS is unavailable (e.g in field studies) or where volumes or concentrations are too low for reliable AAS measurements. 3) There is also a need to measure labile Zn(II) in culture media since many cellular processes are affected by Zn(II). A technique to measure labile Zn(II) in extracellular fluids would enable quantification of Zn(II) release from cells, as well as providing a means to monitor available Zn(II) concentrations in a variety of buffers and media.

For measurement and visualization of labile Zn(II) in cells and fluids we have developed the UV-excitable fluorophore Zinquin (ethyl ester, ZQ). ZQ is sensitive to nM concentrations of free Zn(II), highly specific for Zn(II) amongst other metal cations, and preferentially reactive with the most labile (free or thermodynamically exchangeable) Zn(II) pools.

2. MATERIALS REQUIRED

· TBS-380 Mini-Fluorometer (P/N 3800-003)
· Greiner 1 ml fluorometry cuvettes or mini-cuvettes (Turner Designs)
· Minicell Adaptor Kit (P/N 3800-928)
· Zinquin stock dye solution (Sigma, Calbiochem)
· 10x Zinc-free HBSS buffer stock solution
· High purity water (e.g Milli-Q)

3. FACTORS TO CONSIDER

3.1 The usual precautions are required for minimizing Zn(II) contamination during collection and processing of body fluids.

3.2 To minimize contaminating zinc, use only new disposable plastic ware; Eppendorf tubes can be used for samples and standards and sterile 50 ml polystyrene centrifuge tubes (e.g IWAKI Cat No 2342-050) for buffers; if a large volume of buffer is required, T-75 culture flasks can be used. Note:glass and rubber can introduce zinc ions into solutions. Also use high purity water and chelex-treat buffers (as below).

3.3 EGTA-containing cuvettes are used to obtain the non-labile Zn-dependent fluorescence since the metal chelator EGTA eliminates any labile Zn-dependent ZQ fluorescence. Labile Zn(II) is derived by subtracting mean fluorescence in 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.

3.4 The commercial preparations of Sputolysin, used for dissolving the mucin plugs in sputum plug samples, contain some contaminating Zn(II). To correct for this, on the day of sputum processing, an aliquot of the Sputolysin processing reagent (designated blank) is kept and stored with the samples at -20°C. Zn(II) standards are also prepared in the Sputolysin processing buffer.

3.5 Always remove particulate matter from fluids by centrifugation before adding to cuvettes. This is especially important for thawed plasmas which may contain fibrinogen clots.

3.6 Zinquin fluorescence in cuvettes is linear over the 0-500nM Zn(II) range. Volumes of body fluid added should result in cuvette concentrations of Zn(II) near the mid-point of this range. For some fluids, it may be necessary to dilute first (if concentration is too high) or use a larger volume (if concentration is too low).

3.7 The lower detection limit is 10 pmol of Zn(II) in a 20µl volume (500nM Zn(II) concentration in the biological sample), using mini-cuvettes.

4. SOLUTION PREPARATION

4.1 Zinquin stock dye solution (5mM):
Dissolve 2 mg of Zinquin per mL DMSO and store in aliquots at -20°C. Do not repeatedly freeze-thaw; keep dark.

4.2 10X Zinc-free HBSS buffer stock solution:
Dissolve into 50 mL of Milli-Q water:
0.2 g KCl, MW = 74.55
0.03 g KH2PO4, MW = 136.09
4 g Sodium chloride, MW = 58.44
0.002 g Na2HPO4, anhydrous, MW = 141.96
0.5g D-Glucose, anhydrous MW = 180.16
0.175g NaHCO3, MW = 84.01

Make Zn-free by adding 5g of washed Chelex-100 Resin (per 50 ml buffer) and rotating for 40 min. Remove supernatant and repeat. pH will be ~ 8.6. Do not adjust back to neutral as Zinquin works better at alkaline pH. Store up to 3 months at 4°C.

5. PROTOCOL

NOTE: Accurate pipetting and thorough mixing are critical for reproducible results. However, take extreme care when mixing samples; do not introduce air bubbles. Air bubbles can cause scattering of light leading to inaccurate results. If air bubbles form, hold the upper portion of the cuvette in one hand and gently tap the bottom sides of the cuvette with your other hand to release bubbles.

5.1 Set up unknown sample and Zn(II) standard cuvettes

5.1.1 Method for 1 ml cuvettes

5.1.1.1 Set up 6 cuvettes (Disposable 1 ml fluorometry-grade cuvettes, Greiner) for each unknown sample: 3 without EGTA (for total fluorescence) and 3 with EGTA (for nonspecific fluorescence).

5.1.1.2 Add 20 µl of plasma to each cuvette. For other fluids, see note 3.6.

5.1.1.3 Add 20 µl of 30mM EGTA to the three EGTA cuvettes or water to the without EGTA cuvettes. Ensure the EGTA mixes well with the sample.

5.1.1.4 Set up 3 cuvettes for each Zn(II) standard. Add 20 µl of Zn(II) standard to cuvettes. Use Zn(II) stock solutions of 0, 1.25, 2.5, 5, 7.5, 10, 15, 20 µM Zn nitrate or Zn sulphate. Add 20 µl of water to each cuvette to make volume to 40 µl.

5.1.1.5 Set up 2 cuvettes for calibration of fluorometer. Blank contains 20 µl of 30mM EGTA and calibration cuvette contains 20 ul of 100µM Zn(II).

5.1.1.6 Count the total number of cuvettes and allowing 1 ml of Zinquin-containing buffer for each, calculate the required volume of buffer. Always allow an additional 5%

of volume to avoid running out of buffer during cuvette additions.

5.1.1.7 The following figures assume a volume of 100 ml of buffer (for 95 cuvettes). Adjust accordingly for different numbers of cuvettes. 1) Add 10 ml of 10x Zn(II)-free HBSS (Step 4.2) to 90 ml of Milli-Q water, to prepare a 1x buffer. 2)Add 30 mg ovalbumin (OVA) to give a final concentration of 0.3 mg/ml. OVA is added since it is a Zn(II)-free protein and helps prevent precipitation of the hydrophobic ZQ from aqueous solutions. 3) Immediately before addition of buffer to cuvettes, add 200 µl of Zinquin (5mM in DMSO, step 4.1) to give a final ZQ concentration of 10 µM. Mix well.

5.1.1.8 Add 960 µl of Zinquin-containing buffer to each cuvette.

5.1.1.9 Incubate at RT in dark for a minimum of 40 min. Cuvettes should be read within 2-3 hrs.

5.1.2 Method for mini-cuvettes (useful for fluids containing only nM concentrations of Zn(II))

5.1.2.1 Set up 6 cuvettes (100 µl borosilicate cuvettes, Turner) for each unknown sample: 3 without EGTA (for total fluorescence) and 3 with EGTA (for non-specific fluorescence).

5.1.2.2 Add 25 µl of fluid to each cuvette.

5.1.2.3 Add 25 µl of 30mM EGTA to the three EGTA cuvettes or water to the without EGTA cuvettes. Ensure the EGTA mixes well with the sample and that there are no air bubbles in cuvette.

5.1.2.4 Set up 3 cuvettes for each Zn(II) standard. Add 25 µl of Zn(II) standard to cuvettes. Use Zn(II) stock solutions of 0, 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2 µM Zn nitrate or Zn sulphate. Make volume to 50 µl in cuvettes by adding 25 µl of water.

5.1.2.5 Set up 2 cuvettes for calibration of fluorometer. Blank contains 20 µl of 30mM EGTA and calibration cuvette contains 20 µl of 10µM Zn(II).

5.1.2.6 Count the total number of cuvettes and allowing 0.05 ml of Zinquin-containing buffer for each cuvette, calculate the required volume of buffer. Always allow an additional 5% of volume to avoid running out of buffer during cuvette additions.

5.1.2.7 The following figures assume a volume of 5 ml of buffer (for 95 cuvettes). Adjust accordingly for different numbers of cuvettes. 1) Add 1 ml of 10x Zn(II)-free HBSS (Step 4.2) to 4 ml of Milli-Q water, to prepare a 2x buffer. 2)Add 1.5 mg ovalbumin (OVA) to give a final concentration of 0.3 mg/ml. OVA is added since it is a Zn(II)-free protein and helps prevent precipitation of the hydrophobic ZQ from aqueous solutions. 3) Immediately before addition of buffer to cuvettes, add 20 µl of Zinquin (5mM in DMSO, step 4.1) to give a final ZQ concentration of 10 µM. Mix well.

5.1.2.8 Add 50 µl of Zinquin-containing buffer to each cuvette. Make sure buffer goes to the bottom of the cuvettes and there are no air bubbles.

5.1.2.9 Incubate at RT in dark for a minimum of 40 min. Cuvettes should be read within 2-3 hrs.

5.2 Turn on the fluorometer and select the UV channel. Calibrate the instrument with first the blank cuvette (EGTA) and then the high zinc concentration cuvette (see Step 5.1.1.5 or 5.1.2.5).

5.3 Take readings of all cuvettes. Mini-cuvettes require the adapter with UV facing forward.

5.4. Set up an Excel spreadsheet. For samples, subtract non-specific fluorescence (mean of 3 EGTA + cuvettes) from total fluorescence (mean of 3 EGTA – cuvettes). Convert the fluorescence difference into a labile Zn(II) concentration from the standard curve, allowing for any additional dilution factors made.

6. Table 1 Labile Zn(II) concentrations in some biological fluids using the Turner Biosystems TB380 mini fluorometer.

Fluid	n	Mean Zn (II) μM (+ sem)	% of Total Zn(II)
human plasma	81	8.1 (+ 0.53)	72
human urine	8	0.23 (+ 0.08)	20
human induced sputum	73	1.3 (+ 0.27)	95
human saliva	6	0.11 (+ 0.1)	ND
Mouse plasma	8	4.7 (+ 0.5)	ND
BEGM/DMEM culture medium		1.1 (+ 0.1)	ND

7. References

1. Zalewski PD, Truong-Tran AQ, Lincoln S, Ward D, Shankar A, Coyle P, Jayaram L, Copley A, Grosser D, Murgia C, Lang C and Ruffin RE 2006.Use of a Zinc Fluorophore to Measure labile Pools of Zinc in Body Fluids and Cell-Conditioned Media. Biotechniques Volume 40, Number 4: pp 509-520

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