A fluorescence method to determine picomole amounts of Zn(II) in biological systems

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The formation of complexes between n-(6-methoxy-8-quinolyl)-p-toluensulfonamide (TSQ) and Zn(II) in methanol has been used as an analytical procedure for Zn(II) determination in biological systems. Using 1 ml cuvettes, the limit of detection of the method was approximately 20 pmoles of Zn(II). Linearity between fluorescence and zinc concentration was obtained up to approximately 1 μM Zn(II). Common multivalent cations present in biological systems like Al^{3+}, Cu^{2+}, Fe^{3+}, Ca^{2+}, and Mg^{2+}, interfered with the measurement of Zn(II) only when present in excess of 20, 33, 60, 500 and 30,000 times the Zn (II) concentration, respectively. In human serum and semen, deproteinization of the samples permitted a good correlation between the TSQ method and the total Zn content determined by atomic absorption measurements. In rat spermatids, deproteinization and Zn (II) determination using the TSQ method gives approximately a 20% underestimation of the total Zn (II) content of the cells as compared to atomic absorption spectrophotometry. The method gives low resolution of Zn (II) peaks when tested as an analytical procedure to measure Zn (II) binding to protein fractions eluted during column chromatography.

Key words: *Fluorescence, human semen, human serum, rat spermatids, zinc content, Zn(II).*

INTRODUCTION

Studies investigating the involvement of trace elements in biological functions have necessitated the development of methods for the determination of minute quantities of these elements in complex biological matrices. One such elements, zinc, has important clinical and nutritional implications (1, 11, 12). In addition, the binding of Zn(II) to proteins is also critical because of the importance of this ion in enzyme catalysis, protein and membrane stabilization, and its likely role as a regulatory ion in eukaryotic gene expression (3, 22, 23). Hence, the development of an inexpensive and specific quantitative analytical method for Zn(II) would be helpful.

There are extant several methods for the determination of Zn(II) in biological systems. The most common method of Zn(II) detection has been atomic absorption spectrophotometry (AAS) (9, 15). Although the method is highly specific and sensitive, AAS instrumentation is not always available for routine analysis. Another method developed for Zn(II) determination is based upon enzymatic spectrophotometry that uses *E. coli* alkaline phosphatase. The alkaline phosphatase method combines the biological specificity of the enzyme, its sensitivity, and its availability in most biochemical laboratories (5). The spectrophotometric methods developed for Zn(II) measurement in biological fluids and which use the ion-chelating

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properties of some compounds (10, 16, 18) have the drawback of non-specificity in its basic colorimetric reaction and, hence, require chelating and displacement steps to insure specificity to the determination (e.g., 10).

Cano et *al.* (4) have described a fluorescence methodology for Zn(II) determination using salicylaldehyde thiocarbazone. This method appears to provide enough specificity and sensitivity to permit the detection of Zn(II) in biological fluids. Unfortunately, however, the reagent is not commercially available.

Based on the work published by Fredrickson *et al* (7), Fliss *et al* (6) have studied the interaction of an aqueous suspension of N-(6-methoxy-8-quinolyl)-p-toluensulfonamide (TSQ), with Zn(II) and other metals, providing for a semiquantitative method to measure Zn (II) release from cells proteins. In this paper we describe a fluorescent method that uses the interaction of the commercially available compound TSQ with Zn(II) in methanol that allows for a relatively inexpensive, sensitive and specific determination of Zn(II) in biological fluids. This work was reported in part in abstract form (13).

MATERIALS AND METHODS

Reagents

N-(6-methoxy-8-quinolyl)-p-toluensulfonamide (TSQ) was obtained from Molecular Probes (Eugene,Oregon) and was used without further purification. All the salts were from Merck Chemical Co. (Darmstadt, Germany) or Fisher certified (New Jersey, U.S.A) and were of analytical grade. Methanol was obtained from Riedel de Haen (Seelze, Germany); nitric acid (analytical grade) was purchased from Merck Chemical Co.; and EDTA was obtained from Sigma Chemical Co. (St Louis, MO.). Histidine and cysteine were obtained from Fluka Chemika (Switzerland). Glass distilled and deionized water was used throughout the study. Stock standard Zn(II) solutions were certified from Merck or were prepared in distilled deionized water from zinc sulfate heptahydrate (analytical grade, Merck Chem. Co.), and standardized using AAS. Cadmium working standard solutions were prepared from dilutions of a certified standard (Merck Chem. Co.).

Apparatus

All glass materials were washed with a 1 M nitric acid solution. A Perkin-Elmer MPF 44B spectrofluorometer was used for spectrofluorimetric Zn(II)-TSQ studies, and an Aminco SPF 125 or a Farrand fluorometer was used for routine determinations of fluorescence.

Determination of Zn(II) in biological materials

Quantitative determination of Zn (II) using the TSQ method. Daily calibration of the fluorimeter was accomplished using methanol as blank and 500 nM quinine sulfate in 0.2 M sulfuric acid as 100% scale. The fluorescence of 2 ml of a stock solution of 50 μM TSQ in methanol (prepared fresh daily) was determined in order to estimate basal contaminations. Readings of less than 15% full scale were considered acceptable for further use of the stock 50 μM TSQ solution. 100 μM of the sample was added to 2 ml TSQ-methanol solution in the fluorimeter cuvette, and a sample reading was obtained. Successive additions of Zn(II) standard *after* the sample allowed for the estimation, in the presence of any interferences in the sample, of the fluorescence (F) *vs* Zn(II) concentration slope, as well as to check for the identity of the y intercept of the regression line with the sample reading. In general, the slope of F *vs* Zn(II) concentrations curves in the presence of the samples assayed in this work differed by 30% or less from the control slopes in the absence of sample. The concentration in the sample was calculated using the slope of the F *vs* Zn(II) concentration curve, after subtracting the TSQ-methanol reading from the sample reading.

Human serum. Serum batches from Carlos Van Buren Hospital, Valparaíso, were either directly used for Zn(II) measurements using AAS (15), or deproteinized with 4% trichloroacetic acid (TCA) for TSQ-Zn(II) determinations. For Zn(II) determinations using

the method presented here, the samples were first neutralized with Tris-NaOH (0.7 M each).

Rat spermatids. Spermatids were prepared from testicles obtained from 300 g rats. The testicles were treated with collagenase, and the resulting seminiferous tubules were subjected to mechanical disruption. Rat spermatids were separated in a continuous, nonlinear Percoll gradient as previously described (8). The cell suspension was treated with 1 μg/ml DNAse and 0.1% Triton X-100. The cell homogenate Zn(II) content was determined directly from a dilution of the homogenate using AAS, or after deproteinization with 4% TCA using the TSQ method described in this paper.

Human seminal fluid. Semen samples from healthy males were obtained from a local infertility clinic in Valparaiso. After incubation for 20 minutes at 37°C, the spermatozoa were pelleted by centrifugation at 10,000 x g for 5 minutes, the supernatant was removed and stored at -20° C until zinc determinations were performed. Zinc content was determined directly from a dilution of the seminal plasma or after deproteinization with 4% TCA using AAS, or the TSQ method reported here.

Protein chromatography. In order to explore the usefulness of the TSQ method to resolve Zn(II) binding protein profiles separated by chromatography, we have compared the distribution of Zn(II) using the TSQ method with that of 65Zn(II) in gel chromatography eluates. Protein chromatography was performed using Fractogel TSK HW-55 (Merck Chemical Co.) in a 17 x 790 mm column. Purified proteins or liver extract were applied to the column in 0.1 M Tris-HCl buffer, 0.2 M KCl, pH 8.1, and 4 μ Ci of ^{65}Zn(II). The chromatography was run at 67 ml/h, and fractions of 4 ml were collected. ^{65}Zn(II) was determined by liquid scintillation counting. Zn(II), using the TSQ method described here, were determined after acid deproteinization of the samples.

Contamination control. Common nuisances in trace element determinations are the basal elemental content of the reagents or vessel and/or operator contamination. Thus, a certified contamination of less than 0.00001 % w/v Zn(II) in methanol could produce contaminations of >500 nM Zn(II) in the assay. We routinely checked for contamination testing the fluorescence of the reagents produced by 50 μM TSQ and running blank deproteinizations. All materials used in our determinations were either plastic dispossables tested for contamination in each batch, or glass that was routinely incubated and washed with 1 M nitric acid.

Statistical analysis. The data were first tested for homogenous variance (F test) and subsequently tested by a two tailed Student (t test) as the difference of the mean of two groups or as the difference of the slopes after regression analysis (20).

RESULTS AND DISCUSSION

TSQ-Zn(II) fluorescence

The uncorrected excitation and emission spectra of TSQ-Zn(II) complexes obtained at different Zn(II) concentrations with 5 μM TSQ in methanol are shown in Figure 1. The peak excitation and emission wavelengths are 367 and 495 nm, respectively. The Stokes shift is approximately 120 nm with no overlapping of excitation and emission spectra.

The most common cations in biological systems, *i.e.*, Na^+, K^+, Mn(II), Ca(II), Mg(II), Fe(III), Cu(II), Hg(II), and Al(III), tested up to concentrations of 10, 10, 1.4, 1, 1, 0.02, 0.01, 0.018 and 0.012 mM, respectively,

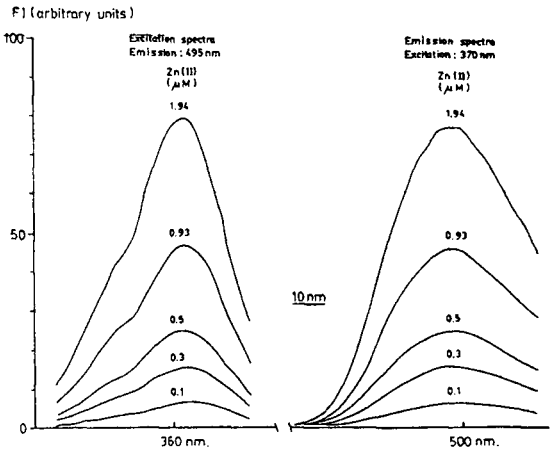

Fig 1: Excitation and emission spectra of 5 μM TSQ in methanol at different total Zn(II) concentrations. Excitation slit: 5 nm. Emission slit: 5 nm. 20° C. Ordinate: Fluorescence intensity (FI) in arbitrary units.

gave no fluorescence upon addition to 5 μM TSQ in methanol. Of all the cations tested, only Cd(II)-TSQ gave a fluorescence comparable to Zn(II)-TSQ (Fig 2).

Quenching of TSQ-Zn(II) fluorescence by inorganic cations

Cu(II), Fe(III), Mn(II), Hg(II), Ca(II), Mg(II), and Al(III) effectively quench the fluorescence of TSQ- Zn(II). Figure 3a shows the concentration- dependence of this quenching effect using 5 μM TSQ and 8 μM Zn(II) in methanol. Because of the high concentrations of Na^+ and K^+ at which a similar quenching effect is observed (10 and 20 mM, respectively), we cannot exclude the possibility that the quenching effect observed with Na^+ and K^+ could be due to trace contamination by other multivalent cations like those described above. Increasing the TSQ concentration by a factor of 10 produces a 3-fold right shift in the concentration-dependence of the quenching by Cu(II) (Fig 3b). A similar 3-fold right-shift in the quench curve after increasing the TSQ concentration to 50 μM was also seen using the other interfering multivalent cations (not shown). Therefore, in this work, we chose a 50 μM TSQ concentration for analytical applications of Zn(II)-TSQ fluorescence.

Linearity of fluorescence and Zn(II) concentration and analytical interferences

The relation between fluorescence intensity and Zn(II) concentrations at low Zn(II) concentrations (*i.e.*, <600 nM) with 50 μM TSQ is shown in Figure 4. This relationship indicates that TSQ-Zn(II) fluorescence can, in principle, be used to quantitatively estimate Zn(II). This linear relationship between TSQ-Zn(II) fluorescence obtains up to at least 1 μM Zn(II) in the assay. Figure 4 also shows the standard deviations and coefficients of variation for the determinations of standard concentrations of Zn(II) between 50 and 600 nM in the assay. Increasing instrument sensitivity and using lower Zn(II) concentrations, gave variation coefficients that were 25, 12, 10, and 4.4% at 9.9, 19.7, 29.4, and 48.5 nM [Zn(II)], respectively.

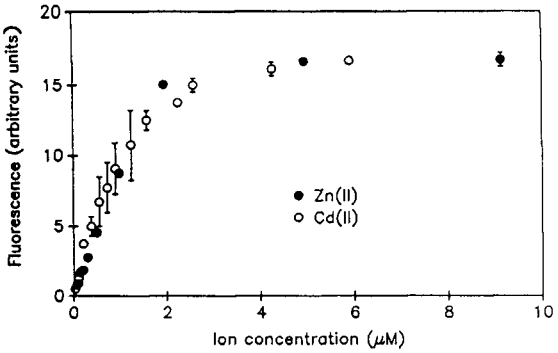

Fig 2: Cation concentration dependence of the fluorescence of 5 μM TSQ in methanol. Other conditions are similar to Figure 1. The abscissa shows total Zn(II) or Cd(II) concentrations in the assay.

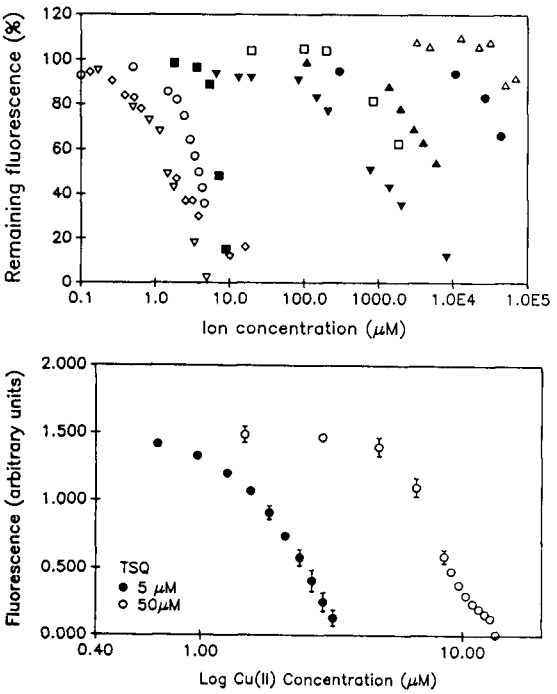

Fig 3: a: Quenching of TSQ-Zn(II) fluorescence by di- and trivalent cations. 100 % fluorescence intensity defined as fluorescence given by 5 μM TSQ-8 μM Zn (II). Symbols: Cu^{2+}; Hg^{2+}; Al^{3+}; Fe^{3+}; Mn^{2+}; Ca^{2+}; Mg^{2+}; Na^+; K^+.
b. Cu(II) quenching curves of the TSQ-Zn(II) fluorescence at two different TSQ concentrations (5 and 50 μM). Zn(II) concentration was 8 μM.

Cation interference or the presence of competing Zn(II)-chelating compounds in the assay medium can be detected by a decrease in the slope of the fluorescence intensity (F) *vs* [Zn(II)] regression line. The maximal ion concentrations at which Al(III),

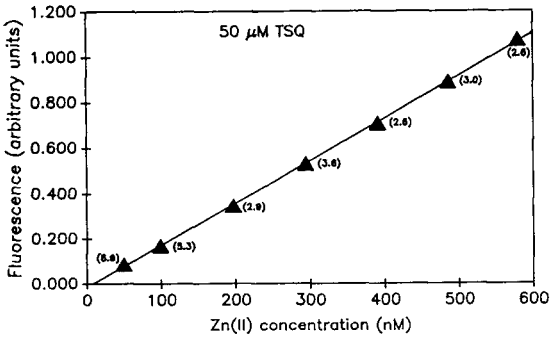

Fig 4: Calibration curve of TSQ-Zn(II) fluorescence at different total Zn(II) concentrations in the assay medium. TSQ concentration was 50 μM in methanol. Numbers in parentheses, the coefficient of variation at each Zn(II) concentration from 5 independent determinations. Temperature was 20° C.

TABLE I

Maximal cation concentrations not affecting sensitivity in the TSQ-Zn standard curves

Cation	Concentration (μM)
magnesium	3,000
calcium	50
iron (III)	6
copper(II)	3
aluminum(III)	2

The concentrations of ions listed produce insignificant decreases in slopes of Zn(II) standard curves with 50 μM TSQ ($p > 0.10$). Higher concentrations of ions significantly decrease slope of the Zn(II) standard curves ($p < 0.05$).

Cu(II), Fe(III), Ca(II), and Mg(II) did not decrease significantly ($p > 0.10$) the slope of the F *vs* [Zn(II)] curve are listed in Table I. Hence, at 100 nM in the assay medium, Zn(II) can be measured in the presence of 20, 33, 60, 500 and 30,000 times excess of Al(III), Cu(II), Fe(III), Ca(II) and Mg(II), respectively. In human serum, the upper reference level of Al (III), Cu(II), Fe(III), Ca(II) and Mg(II) is in the ratio of 0.03, 2.1, 2.8, 238 and 91 with respect to Zn(II) (21), suggesting that practically no interferences are expected from these cations in the concentrations that are normally present in biological fluids such as serum, plasma, cerebrospinal fluid or semen.

Addition of cysteine, histidine or EDTA in the assay medium effectively suppressed the formation of TSQ-Zn(II) complexes in methanol with 50 μM TSQ, and 200 nM Zn(II) (Fig 5). The inflections of the FI *vs* concentration curves for cysteine and histidine most likely reflect the different stoichiometries of the cys-Zn(II) and his-Zn(II) complexes. These data indicate that chelating substances that bind Zn(II) with high affinity can potentially interfere with the determination of Zn using this Zn(II)-TSQ fluorescence method. In the determination of serum zinc, serum cysteine and histidine are expected to reach approximately 0.6 and 1.2 μM, respectively, in the assay medium (17).

The absorbance of 50 μM TSQ and up to 500 nM Zn(II) in methanol is less than 0.02 units. These data and the large Stokes shift of the TSQ-Zn(II) fluorescence indicate that a negligible inner filter effect is expected under these standard working conditions (2).

Properties of TSQ-Zn(II) and TSQ-Cd(II) binding in methanol

Using the molar ratio method for the study of the stoichiometry and stability of complex ions in solution (19), and assuming no changes in quantum yield at different molar ratios, we have estimated a stoichiometry of Zn(II) and Cd(II) binding to TSQ of 1.11 ± 0.39 (n = 7) and 1.21 ± 0.35 (n = 3), respectively. The dissociation constants for the Zn(II)-TSQ and Cd(II)-TSQ complexes determined with the above mentioned procedure were 1.42 (± 0.45) x 10^{-8} M (n = 7) and 2.78 (± 0.98) x 10^{-8} M (n = 3), respectively. The stoichiometry of the Zn (II)-TSQ complex is dependent on the acid-base status of

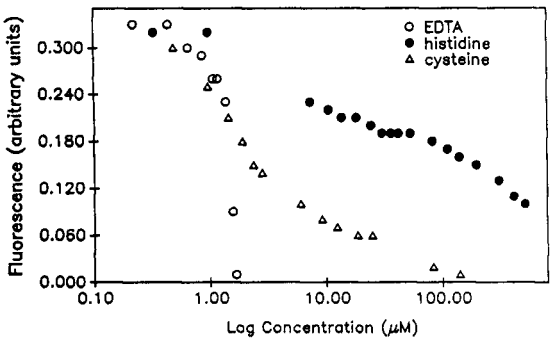

Fig 5: Effects of EDTA, histidine and cysteine on TSQ-Zn(II) fluorescence. Experimental conditions as in Figures 1 and 3. TSQ 50 μM; Zn(II) 200 nM.

the medium. Addition of Tris-HCl, pH 8.5, at a final concentration of 50 μM allows an estimation of the 1:1 stoichiometry estimated above, either by titrating Zn(II) with TSQ or vice versa. However, in the absence of the base, the stoichiometry estimated for the Zn(II)-TSQ complex in methanol is 1.8 ± 0.45 (n = 3) (see also Fig. 2).

Dependence of TSQ-Zn(II) fluorescence on sample pH

Using blanks, deproteinized in the same fashion as serum and titrated with 0.7 M Tris- 0.7 M NaOH, we tested the effects of sample pH on the development of Zn(II)-TSQ fluorescence. Figure 6 shows that acid pH values decreased the slope of the fluorescence *vs* zinc concentration curves. At sample pH's higher than 7 the slope of the standard curves become independent of pH, at least up to pH 9.0.

Zn(II) in human serum, semen and rat spermatids

The Zn(II) content of human serum, semen and rat spermatids was determined using both AAS and the TSQ method. Exogenous zinc was added to different batches of human serum in order to obtain a range of Zn(II) concentrations up to 35 μM. Figure 7 shows that a good correlation existed between the values obtained using AAS and the TSQ method. At concentrations higher than 25 μM Zn(II) in serum, acid deproteinization appears to precipitate an insoluble Zn(II) salt, because the curve becomes flattened with respect to the Y axis at high Zn(II) concentrations. This problem can be overcome by dilution of the high Zn(II) sample and performing deproteinization and Zn(II) determinations in the diluted sample (not shown). The determinations using AAS and TSQ in a deproteinized extract from rat spermatids were 1.8 ± 0.2 and 1.7 ± 0.3 nmoles/mg protein, respectively. However, the total Zn(II) content in the rat spermatids, determined using AAS without deproteinizing the sample, was 2.21 ± 0.2 nmoles/mg protein, indicating that approximately 20% of the total cell Zn(II) is removed with the TCA precipitate in this preparation.

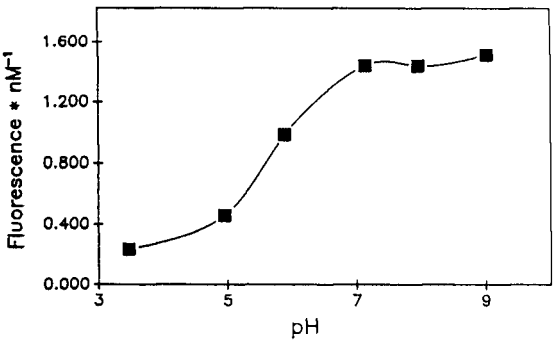

Fig 6: pH dependence of the slope of Zn(II) calibration curves (see Fig 3b) at 50 μM TSQ. Standard errors of the slopes were smaller than symbols shown.

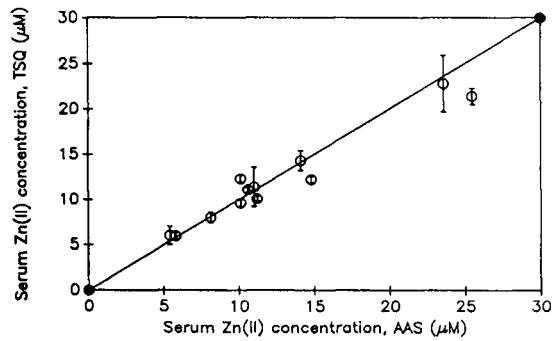

Fig 7: Comparison of serum Zn(II) concentrations using TSQ method (ordinate) and atomic absorption spectroscopy (abscissa). Solid line, slope of 1. The linear regression parameters for the data are: intercept: 1.81 ± 0.83 μM, slope: 0.82 ± 0.06.

In one human semen sample, the values obtained using AAS or the TSQ method after semen deproteinization were 2.9 ± 0.2 and 3.1 ± 0.2 mM, respectively. A comparative study of Zn(II) determination in two semen samples, using either a dilution of the seminal fluid or dilution and acid deproteinization, gave values of 1.54 ± 0.13, 1.80 ± 0.08 (dilution), and 2.05 ± 0.12, 2.26 ± 0.09 (dilution - deproteinization), respectively. A paired t-Student analysis shows that the values are significantly different at the level $p < 0.005$. Hence, simple dilution of the semen would underestimate by approximately 20-25% the total Zn(II) concentration.

Determination of Zn(II) associated with protein fractions in gel chromatography

We have compared the elution profiles of Zn(II) content determined with TSQ and the

distribution of radioactive $^{65}Zn(II)$ in order to validate the TSQ method as a screening test in protein chromatography of Zn(II)-binding proteins. The profile of $^{65}Zn(II)$ binding and of Zn(II) concentrations in the eluate of a chromatography of cytosolic liver homogenate and purified carbonic anhydrase is shown in Figures 8a and b. The profiles of both methods to determine Zn(II) binding show a similar pattern. The chromatography profile of the cytosolic fraction liver homogenate shows the binding of Zn(II) to hemoglobin (14) which was identified by absorbance at 580 nm, and most likely, to carbonic anhydrase which elutes in approximately the expected region as determined in purified standards (Fig 8b). No special effort was made to separate metalothionein, which appears to be eluting close to the free zinc peak in our column conditions (not shown). Undoubtedly, the sensitivity and resolution of radioactive determinations is much better than the TSQ-Zn(II) method which has a limit of detection of approximately 0.5 μM in the column eluate (due to dilution of the sample with deproteinization and neutralization).

The method described in this work can measure Zn(II) that is released from protein binding sites by TCA protein denaturation or Zn(II) bound to binding sites with affinities much smaller than TSQ. This seems to be the reason for the discrepancy between total Zn(II) content in rat spermatids and Zn(II) content determined after deproteinization, as well as the differences observed in zinc concentration in semen samples diluted or diluted and deproteinized. In principle, the methodology to release Zn(II) from protein binding sites could be modified and improved, the limiting factors being the need to avoid additions of Zn(II) chelators at concentrations that could compete with TSQ, and the use of salts containing Zn(II) at contaminating levels. Use of higher TSQ concentrations could also increase the capacity of TSQ to remove and quantify Zn(II) bound to high and low molecular ligands in biological fluids. Nevertheless, this TSQ fluorescence method affords an easy, efficient, sensitive, and inexpensive way to measure Zn(II) in biological fluids, especially when additions of standards are applied after the

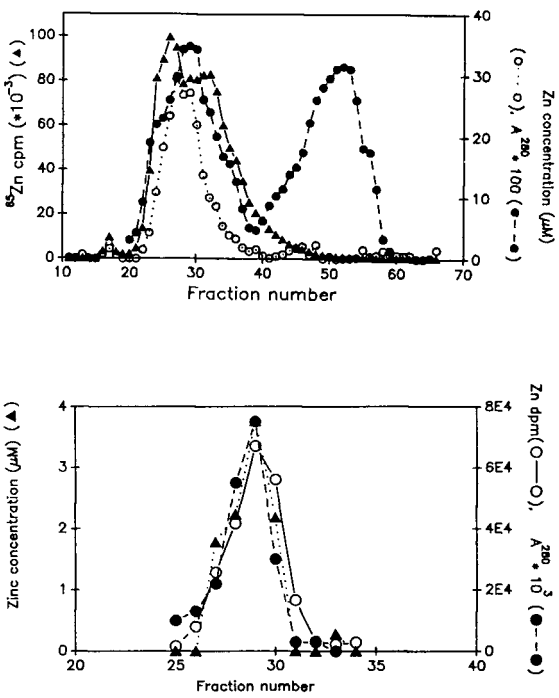

Fig 8: Fractogel TSK - HW55 elution profile of Zn(II), determined with the TSQ method and 65Zn(II) radioactivity: a. Liver homogenate (50 mg protein). b. Purified carbonic anhydrase (5 mg protein).

samples, to account for the likely presence of interferences in biological fluids.

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