Optimized Atomic Absorption Spectrophotometry of Zinc in Cerebrospinal Fluid

R. Palm, R. Sjöström, and G. Hallmans

This method for direct determination of Zn in cerebrospinal fluid (CSF) involves flame atomic absorption spectrophotometry with a pulse nebulizer technique. Standard solutions of Zn in 150 mmol/L NaCl were used. We could account for 88% of added standard with the method in individual samples from 10 patients and in pooled CSF. The method is acceptably precise, CVs in pooled CSF ranging from 4 to 12%. The mean CSF-Zn concentration for nine healthy men was 0.18 (SD 0.04) μmol/L and for nine healthy women 0.15 (SD 0.03) μmol/L, a statistically insignificant difference. These values are lower than those in previous reports, which may have been the result of contamination problems, nonatomic absorption, or nonstandardized sampling. In the healthy volunteers, the CSF-Zn concentration was positively correlated with serum-Zn, CSF-protein, and CSF-albumin concentrations, as well as with the CSF/serum ratio for albumin.

Additional Keyphrases: reference interval, trace elements

There are many reports on the normal concentration of zinc in serum or plasma (1) and how it is influenced by various diseases (2), age and sex (3), diurnal variations (4), pregnancy and oral contraceptives (5), and therapy with corticosteroids (6) and corticotropin (7). Low concentrations of Zn in plasma or serum have been found in multiple sclerosis (8, 9) and chronic alcoholism (10), high concentrations in Pick’s disease (11) and multiple sclerosis (12). The importance of these changes for the etiology or pathogenesis of the lesions in the central nervous system is unknown.

Reports of zinc concentrations in the cerebrospinal fluid (CSF-Zn) are few, and the results (Table 1) are variable (13–21). Different analytical methods have been used, including neutron activation analysis and flame or flameless atomic absorption spectrophotometry. No previous reports have been published on Zn concentrations in CSF from healthy volunteers as assayed after well-controlled sampling procedures.

We wanted to optimize the flame atomic absorption spectrophotometry method for the determination of CSF-Zn concentrations, using as few analytical steps as possible so as to minimize the risks of contamination. In addition we measured Zn in CSF and serum from healthy volunteers, with use of a well-controlled sampling procedure. The relationships between CSF-Zn, CSF-protein, CSF-albumin, and serum-Zn were also determined.

Materials and Methods

Patients and Experimental Design

Four tests—A, B, C, and D—were performed. In tests A, B, and C, CSF was obtained from patients in whom a spinal tap was performed for diagnostic reasons; in test D, CSF came from healthy volunteers. The zinc concentrations in CSF were determined by atomic absorption spectrophotometry, either directly ("direct method") or after known amounts of Zn were added to the CSF ("standard-addition method").

In test A, CSF from one patient was used to estimate the background (non-atomic) absorption.

In test B, CSF from six patients was pooled and used to determine the precision and between-run variation of both the direct method and the standard-addition method. We used three different readout methods (see below) in comparing results by the two methods.

In test C, CSF samples from 10 patients were analyzed by the direct method and the results were compared with those by the standard-addition method as an indirect estimate of the accuracy of the former.

In test D, normal values for CSF-Zn were determined with the direct method in 18 healthy volunteers, nine men (mean age 28 years) and nine women (mean age 30 years). None had clinical or laboratory signs of infection, anemia, liver disease, or pregnancy. None were taking any drugs, including oral contraceptives. They were verbally informed of the aim of the study and all agreed to participate. The project was approved by the ethical committee of the University Hospital.

Methods

CSF sampling. For tests C and D we obtained CSF samples at 0800 hours, after overnight fast. The lumbar puncture was performed with the subject in the lateral recumbent position after a 30-min rest. The skin was anaesthetized with lidocaine, and the puncture was made with a sterile 0.9 x 90 mm disposable hypodermic needle. In test D, the first 2 mL were taken for cell count and CSF samples from the 10th to the 17th mL were used for determination of Zn, total protein, and albumin. CSF was allowed to drop directly into acid-washed plastic tubes, which were immediately sealed with Parafilm and stored at −20 °C until analyzed. The CSF samples were not centrifuged or transferred to other tubes.

Serum sampling. Immediately after the spinal tap, blood samples were taken from an antecubital vein after minimal stasis. The blood was collected in acid-washed glass tubes, allowed to clot, then centrifuged. The serum, transferred to acid-washed plastic tubes with a Pasteur pipette, was stored at −20 °C until analyzed for Zn and albumin.

Instrument

For the zinc determinations we used an AA-6DB atomic absorption spectrophotometer (Varian Techtron Pty Ltd., Melbourne, Australia), equipped with a zinc hollow-cathode lamp, a hydrogen lamp, a 10-cm air acetylene burner, an adjustable tantalum nebulizer, and a Teflon cone for analysis with pulse nebulizer technique (22–24). A strip-chart recorder (Vitatron 2001, The Netherlands) was used for the manual estimation of peak heights; a peak-reader module PRM-6 with printer (L-L Elektronik, Umeå, Sweden) per-
mitted simultaneous digital reading of peak height and peak area (25). A foot pedal was used to zero and to trigger the peak-reader module.

Reagents

**Pure water**: De-ionized pure water, used throughout, was obtained from a "Milli-Q" water purification system (Millipore Corp., Bedford, MA 01730).

**Sodium chloride, 1.5 mol/L**: We used NaCl ("Suprapur" grade; Merck, Darmstadt, F.R.G.).

**Hydrochloric acid, 0.1 mol/L**: Dilute 8.3 mL of concentrated HCl, analytical grade (Merck), to 1,000 L.

**Stock standard solution, 1,000 g/L (15.30 mmol/L)**: Dissolve 1,000 g of Zn in 50 mL of concentrated HCl and dilute to 1,000 L. The final concentration of HCl thus is 0.5 mol/L.

**Intermediate Zn standard, 10 µmol/L**: Dilute the stock solution further to give a zinc concentration of 10 µmol/L.

**Working Zn standards**—0, 0.1, 0.2, and 0.3 µmol/L in 150 mmol/L NaCl for the direct method: Dilute 0, 0.5, 1.0, and 1.5 mL of the 10 µmol/L zinc standard, each plus 5 mL of 1.5 mol/L NaCl, to 50 mL.

**Working standards**—0, 1, 2, and 3 µmol/L for the standard-addition method: Dilute 0, 5, 10, and 15 mL of the 10 µmol/L zinc standard to 50 mL.

Working standards were never more than two days old.

**Glass- and Plasticware**

 Pipettes and volumetric flasks were of borosilicate glass. The 12 × 75 mm plastic tubes were of polystyrene. All plastic tubes and the glassware were acid-washed in two steps:

1. A programmable washing machine with several washing periods was used. The program included washing with a basic surfactant, then with an acid surfactant (containing citric acid), and several rinsings in warm or cold de-ionized water (26).

2. The material was then soaked overnight in 0.1 mol/L HCl, rinsed with pure water, and dried in an oven. After drying, all glass- and plasticware were stored in sealed plastic bags to avoid contamination with dust.

From every set washed, 30 tubes were tested by adding 0.5 mL of 0.1 mol/L HCl to each tube and checking for Zn. If more than 0.01 nmol of Zn was found, the set was re-washed.

**Analytical Procedures**

 The instrument was optimized to minimize background absorption. The operation conditions were as follows: hollow-cathode lamp current 7 mA, monochromator slit width 0.5 nm, and acetylene gas flow 2.5 units (2.2 L/min). Zn absorbance was determined at 213.9 nm. The integration time was 3 s and the absorbance was read to four decimal places. For sample injections into the cone we used a "Finnpipette" (Finnpipette Oy, Finland), adjusted to deliver 100 µL. For each sample we made five injections, each separated by two injections of 0.1 mol/L HCl and one of water. The digital recording of peak height was used in all tests for the registration of absorbance values. In test B, manual measurement of peak height and digital peak area read-out were also used. We rejected the highest and lowest absorbance values obtained for each sample, using the mean of the three remaining values for the calculations. No background correction with the hydrogen lamp was made.

**Background determination.** The background absorbances of CSF, 150 mmol/L NaCl, and water were determined with a hydrogen lamp.

**Direct method.** The samples and standard solutions were injected into the cone and the absorbance values recorded. A standard curve was calculated by using the least-squares method (Figure 1, left). Absorbance values for the different CSF samples were registered and the actual CSF-Zn concentrations calculated from the standard curve.

**Standard-addition method.** CSF samples were divided into four 1-mL portions, and 100 µL of the respective standard solutions was added to each portion. A blank solution consisting of 1 mL of 150 mmol/L NaCl and 100 µL of water was prepared. Absorbances were measured and a regression line was then calculated by use of the least-squares method (Figure 1, right). The CSF-Zn concentration was read from the x-axis at the intercept of the absorbance for the blank.

**Serum-Zn.** We diluted 100 µL of serum with 1.0 mL of 0.1 mol/L HCl. We measured the serum-Zn concentrations with flame atomic absorption spectrophotometry, using the continuous-aspiration technique (26). The mean of two determinations was used in the calculations. As standards, samples of Zn in 0.1 mol/L HCl were used.

**Protein in CSF.** Protein was determined according to Lowry et al. (27), with tyrosine as the standard.

**CSF-albumin and serum-albumin.** These were determined by electrophoimmunooassay according to Laurell (28), with human albumin (Kabi, Sweden) as the standard.

**Statistics.** Differences between group means for different variables were tested by using the Student's t-test for unpaired observations. The test was modified if the variances differed significantly (p < 0.01; F-test). The product moment correlation coefficient (r) was calculated for selected paired variables and tested by Student's t-test.

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**Table 1. Reported Zinc Concentrations in CSF (CSF-Zn)**

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Analytical method</th>
<th>Patients</th>
<th>No. of cases</th>
<th>CSF-Zn, µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Neutron activ. anal.</td>
<td>Psychoneurotic patients</td>
<td>5</td>
<td>0.24 (Range: 0.15–0.31)</td>
</tr>
<tr>
<td>14</td>
<td>AAS (flame)</td>
<td>Psychoneurotic patients</td>
<td>3</td>
<td>0.76 (Range: 0.15–0.31)</td>
</tr>
<tr>
<td>15</td>
<td>AAS (flame)</td>
<td>Patients with serum Zn normal</td>
<td>11</td>
<td>0.61 ± 0.40</td>
</tr>
<tr>
<td>16</td>
<td>AAS (flame)</td>
<td>Patients with convulsions on therapy</td>
<td>26</td>
<td>1.01 ± 1.64 (Range: 0.15–5.35)</td>
</tr>
<tr>
<td>17</td>
<td>?</td>
<td>Patients with tension headache</td>
<td>18</td>
<td>0.46 (Range: 0.15–0.76)</td>
</tr>
<tr>
<td>18</td>
<td>Spark-source mass spectrometry</td>
<td>Patients with neurolog. diseases</td>
<td>27</td>
<td>3.67 ± ?</td>
</tr>
<tr>
<td>19</td>
<td>AAS (flame)</td>
<td>Patients with neurolog. diseases</td>
<td>82</td>
<td>1.13 ± 0.72</td>
</tr>
<tr>
<td>20</td>
<td>AAS (electrotherm.)</td>
<td>Persons without signs of neurolog. diseases</td>
<td>20</td>
<td>2.60 ± 0.64</td>
</tr>
<tr>
<td>21</td>
<td>AAS (flame)</td>
<td>Children with meningism, fever, or headache</td>
<td>30</td>
<td>0.37–0.61</td>
</tr>
<tr>
<td>Present study</td>
<td>AAS (flame)</td>
<td>Healthy volunteers</td>
<td>18</td>
<td>0.14 ± 0.03</td>
</tr>
</tbody>
</table>

*Results expressed as the mean ± SD
Results

Background absorption (test A). The background absorbances of 29 determinations for CSF, 150 mmol/L NaCl, and water were 6.0 (SE 0.1), 6.1 (SE 0.2), and 2.9 (SE 0.1) mA respectively. There were no differences between the absorbances of CSF and of the NaCl solution.

Sensitivity (test B). In test B the sensitivity—i.e., the concentration absorbing 1% of the light—was estimated to be 0.13 μmol/L.

Precision and between-run variation (test B). Precision was somewhat better with the direct method than with the standard-addition method (Table 2), although the difference between the two runs was statistically insignificant.

Read-out methods (test B). There were no significant differences between the mean concentrations obtained with the different read-out methods (Table 2). Digitally recorded peak area showed poorer precision than the manual and digital peak-height readout.

Accuracy (tests B and C). The direct method was compared with the standard-addition method to determine the accuracy of the former. When digital peak-height was used in test B (Table 2), the direct method yielded values that were 91% and 84% of the standard-addition values in two consecutive runs. In test C, the direct method gave values that averaged 88% of the standard-addition values (Figure 2).

Normal values (test D). CSF from all the healthy volunteers was clear and colorless, with a protein concentration of <500 mg/L. There were fewer than five leukocytes and fewer than 350 erythrocytes per microliter in the first 2 mL of the spinal tap. Mean CSF-Zn for the men was 0.16 (SD 0.04) μmol/L and for the women 0.13 (SD 0.02) μmol/L (Table 3). The difference was statistically insignificant. Concentrations of CSF-protein, CSF-albumin, serum-Zn, and serum-albumin were higher in the men. The CSF/serum ratio for albumin showed no sex-related difference. Table 4 lists the correlation coefficients for different variables. CSF-Zn concentrations were related to the concentrations of serum-Zn, CSF-protein, and CSF-albumin as well as to the CSF/serum ratio for albumin. The relation between CSF-Zn and serum-Zn is shown in Figure 3, that between CSF-Zn and CSF-protein in Figure 4.

Discussion

The CSF-Zn concentrations we found, both for patients and healthy volunteers, are lower than in most recent reports (Table 1). Kjellin (13), using neutron activation analysis, reported almost the same values as in the present study. If it is assumed that the CSF-Zn concentrations
Table 3. CSF-Zn, Serum-Zn, CSF-Protein, CSF-Albumin, Serum-Albumin Concentrations and CSF/Serum Ratio for Albumin in 18 Healthy Volunteers

<table>
<thead>
<tr>
<th>Age, years</th>
<th>CSF-Zn</th>
<th>Serum Zn</th>
<th>CSF-protein</th>
<th>Serum albumin</th>
<th>CSF/S albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (n=9)</td>
<td>28 ± 4</td>
<td>0.16 ± 0.04</td>
<td>15.8 ± 1.2</td>
<td>365 ± 62</td>
<td>178 ± 35</td>
</tr>
<tr>
<td>Women (n=9)</td>
<td>30 ± 4</td>
<td>0.13 ± 0.02</td>
<td>16.0 ± 1.2</td>
<td>289 ± 48</td>
<td>134 ± 30</td>
</tr>
<tr>
<td>Men + women (n=18)</td>
<td>29 ± 4</td>
<td>0.14 ± 0.03</td>
<td>14.9 ± 1.4</td>
<td>327 ± 66</td>
<td>156 ± 39</td>
</tr>
</tbody>
</table>

*Results are given as the mean ± SD; p-values are for the difference between values are for men and women.

Table 4. Coefficients of Correlation between CSF-Zn and Serum-Zn, CSF-Protein, CSF-Albumin, and CSF/Serum Ratio for Albumin in 18 Healthy Volunteers

<table>
<thead>
<tr>
<th></th>
<th>CSF-Zn</th>
<th>CSF-Zn-</th>
<th>CSF-Zn-</th>
<th>CSF-Zn-CSF-</th>
<th>CSF-Zn-CSF/</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Zn</td>
<td>r = 0.75</td>
<td>r = 0.75</td>
<td>r = 0.37</td>
<td>r = 0.58</td>
<td></td>
</tr>
<tr>
<td>CSF-Protein</td>
<td>p = 0.018</td>
<td>p = 0.018</td>
<td>p = 0.327</td>
<td>p = 0.100</td>
<td></td>
</tr>
<tr>
<td>CSF-Albumin</td>
<td>r = -0.26</td>
<td>r = 0.59</td>
<td>r = 0.56</td>
<td>r = 0.84</td>
<td></td>
</tr>
<tr>
<td>Serum Albumin</td>
<td>p = 0.497</td>
<td>p = 0.094</td>
<td>p = 0.116</td>
<td>p = 0.062</td>
<td></td>
</tr>
<tr>
<td>Men (n=9)</td>
<td>r = 0.50</td>
<td>r = 0.76</td>
<td>r = 0.57</td>
<td>r = 0.66</td>
<td></td>
</tr>
<tr>
<td>Women (n=9)</td>
<td>p = 0.034</td>
<td>p &lt; 0.001</td>
<td>p = 0.013</td>
<td>p = 0.003</td>
<td></td>
</tr>
<tr>
<td>Men + women (n=18)</td>
<td>r = 0.50 (p = 0.034)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The highly variable results obtained by others might be explained on the basis of contamination problems, background absorption, nonstandardized CSF sampling, and use of different analytical methods. The contamination problems are considerable in zinc analysis (29), in view of the very low concentrations found in CSF. Analytical procedures that require, e.g., the addition of reagents, ion-exchange chromatography, and the transferring of samples between different tubes increase the contamination risks. To minimize contamination risks in the present study, the CSF was not centrifuged and the samples were kept in the same tube until analyzed. The washing procedures of the laboratory utensils are also very important. The equipment used must be completely free of measurable zinc.

CSF-Zn concentrations will be increased by Zn from erythrocytes if a spinal tap is traumatic. Erythrocytes have a relatively high content of Zn, $14 \times 10^{-12}$ μmol per
erythocyte (30). Cerebrospinal fluid will be macroscopically clear when there are fewer than 500 erythrocytes per microliter of CSF (31, 32). However, 700 erythrocytes per microliter of CSF will, theoretically, add only 0.01 μmol of Zn per liter to the CSF if erythrocyte hemolysis is complete. This will not significantly alter the observed CSF-Zn concentration. Thus we considered macroscopically clear and colorless CSF satisfactory as a sample. Additionally, CSF-Zn was analyzed in samples obtained late in the spinal tap, to minimize contamination with blood.

In Zn determinations by atomic absorption spectrophotometry the background absorption, due mainly to salts, is also a problem. Sodium, the principal cation in CSF at a concentration of 143 mmol/L (33), has a pronounced absorption at the Zn resonance line (34). Background corrections have not been common in previous reports and were not made in the present study because the noise level increases as the hydrogen lamp is used, increasing the detection limit. Without the background correction all peaks were greater than the noise level, and the analytical procedure was simpler. We decreased the background absorption to a low and acceptable level by optimizing the instrument. NaCl was used to compensate for the background absorption, because CSF and 150 mmol/L NaCl had the same background correction.

One advantage of the pulse nebulizer technique over continuous aspiration into the flame is the smaller sample volume requirement. Thus, five determinations instead of one were possible from the same volume when this technique is used, with less likelihood of system clogging (35). Lower CSF-Zn concentrations were found with the direct method than with the standard-addition method. Differences in nebulization rate between CSF and the standards may explain this discrepancy. The precision was found to be the same in manual and digital peak-height readout in test B. The latter method, which is objective and simple, was used for the readouts in the succeeding tests.

Serum-Zn concentrations for the healthy volunteers were within the normal range (1, 36), and were significantly higher for men than women, as also reported by some authors (3, 36) but not by others (37, 38).

Values for CSF-protein and CSF-albumin were higher for men. This sex-related difference was also seen by Breebaart et al. (39) but not by others (40). In previous reports Kjellin (13) found a positive relation between CSF-Zn and CSF-protein (in three patients with high CSF-protein concentrations), but other authors did not (15, 19, 21). In the present study, we found significant correlations between CSF-Zn and CSF-protein, between CSF-Zn and CSF-albumin concentrations, and between CSF-Zn and the CSF/serum ratio for albumin. The latter ratio is regarded as a good test of the blood–brain barrier function (40).

We also found a weak positive correlation between CSF-Zn and serum-Zn concentrations in the men (Figure 4). Woodbury et al. (15) did not find this relation.

In serum, Zn is bound mainly to albumin (about 80%) and to α2-macroglobulin (about 20%) (41). A small part is also bound to amino acids such as histidine and cysteine (42). It is not known if Zn in CSF is bound to proteins. CSF-albumin derives from serum-albumin (43) and the concentration of α2-macroglobulin in CSF is very low (44). Therefore CSF-Zn probably is bound mainly to albumin. CSF has, however, a relatively high concentration of amino acids. The serum/CSF ratio for albumin is about 200:1 (40) and for histidine 6:1 (45), which suggests the possibility of a proportionately greater binding between Zn and amino acids in CSF.

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References
Improved Measurement of Acetylsalicylic Acid Esterase in Serum

Steen Sørensen

A fixed-time incubation method for measuring acetylsalicylic acid esterase was improved by using a higher concentration of the substrate, acetylsalicylic acid, and an activator, Ca\(^{2+}\). The enzymatic activity in serum was 25-fold that measured by earlier methods. Inhibition studies showed a pattern similar to that reported for cholinesterase. Use of cholinesterase inhibitor in sample tubes for determination of acetylsalicylic acid and salicylic acid is recommended.

Additional Keyphrases: aspirin  ·  carboxylic ester hydrolases  ·  cholinesterase inhibitors  ·  enzyme tests  ·  reference interval

Owing to its analgesic, anti-inflammatory, and anti-thrombotic effects, acetylsalicylic acid (ASA, aspirin) is one of the most commonly used drugs. After oral administration, ASA is hydrolyzed to salicylate (SA), either in intestinal fluids, during passage across the gastrointestinal wall, or during the first passage of the drug through the liver (1). The absorption, distribution, and elimination pharmacokinetics of ASA and SA have recently been examined (2). Acetylsalicylic acid esterase (ASA esterase), a species of carboxylic ester hydrolase (EC 3.1.1) catalyzes hydrolysis of ASA to SA in blood, but methods for measuring ASA esterase (3–6) are poorly optimized.

I have improved this assay by using a higher substrate concentration and adding Ca\(^{2+}\) as an activator. As a result the ASA esterase activity measured in normal sera was 25-fold that earlier reported. I also carried out inhibition tests for comparison with similar published studies of other esterases in human serum.

Materials and Methods

Reagents. Acetazolamide, acetylsalicylic acid, ecohthiophate, neostigmine bromide, and salicylic acid were of pharmacopeial quality; the other reagents were of analytical grade.

Buffer. Tris HCl buffer, 300 mmol/L, pH 7.6–7.7 (20 °C), containing CaCl\(_2\), 200 mmol/L.

Standard. Salicylic acid, 9.05 mmol/L (1.25 g of salicylic


