Fluorimetry of Selenium in Body Fluids after Digestion with Nitric Acid, Magnesium Nitrate Hexahydrate, and Hydrochloric Acid

Jean Pettersson, Lena Hansson, Ulf Önemark, and Åke Olén

A digestion procedure involving nitric acid, magnesium nitrate hexahydrate, and hydrochloric acid suffices for selenium determinations in whole blood, serum, and urine by molecular fluorescence spectrometry. To test the accuracy of the method we compared the results with those from hydride-generation atomic absorption spectrometry, and we also analyzed reference materials.

Additional Keyphrases: trace elements · molecular fluorescence spectrometry · hydride-generation atomic absorption spectrometry compared · urine

About half of those laboratories involved in selenium determinations in blood perform their analyses by molecular fluorescence spectrometry (MFS) (1, 2). The procedure is usually based on the reaction between tetravalent selenium and 2,3-diaminophenanthrene at a pH between 1 and 2. A fluorescent complex, 4,5-benzopiazelenol, is formed, which is extracted into an organic phase (usually cyclohexane), where its fluorescence is measured. Most often, the sample digestion is performed with an acid mixture including perchloric acid (e.g., 3). Efforts to replace this hazardous but effective agent for destruction of biological material include trials of a combination of phosphoric acid, nitric acid, and hydrogen peroxide (4). A digestion procedure based on a mixture of magnesium nitrate, nitric acid, and hydrochloric acid for the determination of selenium in blood and urine samples has proved suitable when combined with hydride-generation atomic absorption spectrometry (HG-AAS) (5). Here we report the compatibility of this digestion procedure with the well-established MFS method for determining selenium.

Materials and Methods

Apparatus

We used the HG-AAS instrumentation described previously (6), a fluorescence spectrometer (Perkin-Elmer Model 204) equipped with a xenon lamp, and 1.0-cm (pathlength) quartz cuvettes for all measurements.

A temperature-regulated aluminum block (6) was used for digestion, reduction, and reaction.

Reagents and Materials

All acids and reagents were of "pro analysi" quality except for the diaminonaphthalene (Fluka Chemie AG, Buchs, Switzerland), which had to be purified just before use as follows. To 50 mL of de-aerated 0.1 mol/L HCl add 50 mg of diaminonaphthalene and 250 mg of hydroxylation chloride. Heat this mixture at 50 °C for 15 min, cool, and filter the solution into a separatory funnel. Add 10 mL of cyclohexane, shake for 1 min, and let the phases separate for 5 min. Filter the aqueous (lower) phase into a second funnel containing 10 mL of cyclohexane and repeat the extraction. This time, filter the aqueous phase into a 50-mL screw-capped test tube. Bubble nitrogen gas through the mixture for 2 min, and cap the tube.

The indicator solution was prepared by dissolving 0.3 g of gentian violet in 100 mL of water; the 36 mmol/L EDTA solution was prepared by dissolving 6.7 g of Na₂EDTA · H₂O in 500 mL of water.

Selenium standard solutions were prepared as described in ref. 6.

The reference materials were Standard Reference Material no. 1577a (bovine liver; U.S. National Bureau of Standards, Gaithersburg, MD) and Seronorm 105 (human serum) and 108 (human urine) from Nycomed AS, Oslo, Norway.

The filter paper used was of medium pore size (no. 00M; Munktell, Grycksbo, Sweden).
Procedure

Digestion. Weigh the sample (about 0.1 g of lyophilized blood, 1 g of serum, or 1 g of urine) into a 50-mL screw-capped test tube. Add 4 g of Mg(NO₃)₂ · 6H₂O, 10 mL of concentrated nitric acid, and 1 mL of 6 mol/L hydrochloric acid. Heat in an aluminum block in the following sequences of time (h) and temperature (°C): 1–50, 1–85, 1–105, 1–125, 2–175, 2–225, 2–300, and 3–500. Then cool the mixture to room temperature.

Reduction. Add 10 mL of 6 mol/L hydrochloric acid. Cap the test tubes and heat for 1 h at 130 °C.

pH-adjustment. Adjust the hydrogen ion concentration to about 30 mmol/L by first adding 3.5 mL of 6 mol/L ammonia reagent, then 0.5 mL of the indicator, 1 mL of 36 mmol/L EDTA, and finally ammonia reagent (2 mol/L solution) until a green color is visible.

Reaction and extraction. Bubble nitrogen gas through the digested and reduced sample for 30 s, then add 1 mL of diaminonaphthalene reagent and 5 mL of cyclohexane. Cap the test tube and place it for 30 min in the aluminum block, which has been preheated to 60 °C. Cool to room temperature, shake for 1 min, and transfer the organic layer to a test tube after 5 min. Measure the fluorescence of the sample (excitation wavelength 375 nm, emission wavelength 522 nm).

Results and Discussion

The reduction of selenate to selenite. After digestion, one must be sure that all the selenium in the sample is in the tetravalent state. This is the only form of selenium that reacts with diaminonaphthalene to give the desired fluorescent species. The reduction is accomplished by reacting the digested sample with hydrochloric acid. If the reaction tubes are open during the reduction, some of the hydrochloric acid added will boil off. This is not a problem in the HG-AAS procedure, but in the MFS method it will cause inconvenience. The temperature varies somewhat with the positions in the aluminum block, and different amounts of hydrochloric acid will leave each test tube. This makes the pH-adjustment in the MFS procedure more time consuming, and the reduction should therefore preferably be performed in closed vessels.

The blank. The diaminonaphthalene reagent is unstable, and its rate of decomposition increases with temperature, exposure to daylight, and presence of oxidizing agents. High and irreproducible blank signals result unless an efficient purification procedure is applied and the purified product is stored under proper conditions. A fluorescing polymer of diaminonaphthalene is held responsible for the undesired blank signals (7). Several purification procedures have been described in the literature (e.g., 8, 9). We found it necessary to purify the diaminonaphthalene reagent by two repeated extractions with cyclohexane, to filter the solution after each extraction, and, last but not least, to deoxygenate the hydrochloric acid used to dissolve the diaminonaphthalene by bubbling with nitrogen gas to ensure low and reproducible blank signals. Hydroxylammonium chloride was also added to the reagent as an antioxidizing agent. Table 1 shows the improvement with the successive purification steps.

The pH adjustment. A pH adjustment is necessary before the complexation reaction between selenite and diaminonaphthalene. A pH of about 1.8 is generally considered optimal (see e.g., 7). The very high salt concentrations obtained after digestion and reduction in the proposed method makes proper pH measurement difficult. Therefore, acidity should rather be expressed as a hydrogen ion concentration than as a pH value. For convenience, however, we use the pH notation understood to mean −log[H⁺]. We found that a pH value between 1 and 2 for the reaction only slightly influenced the fluorescence intensity. Outside these limits the fluorescence declined considerably. The pH adjustment described in the experimental section gives a final pH value of 1.5 ± 0.2, which is sufficiently accurate. It is important that the pH be promptly adjusted after the indicator has been added, because its color begins to fade after about an hour.

Effect of reaction time and temperature. Optimum conditions for the complexation reaction between diaminonaphthalene and selenite were found to be 30 min at 60 °C.

Calibration. The calibration curve was established from known amounts of selenite subjected to the whole procedure, from digestion to fluorimetry. It is, however, not necessary to include the digestion step if all reagents are added to the calibration solutions and the pH is adjusted to the value used for the samples in the complexation step.

Analytical results. Table 2 gives results from the analysis of lyophilized whole-blood samples by fluorimetry and hydride-generation atomic absorption spectrometry. Both MFS and HG-AAS were applied to each digest and all digestions were performed simultaneously. Table 2 also includes the results obtained from analysis of the three reference materials. Results from the two methods overlap, and results for the reference materials were satisfactory.

We conclude that the described digestion procedure is a valid alternative to more common destruction methods (often including perchloric acid) when selenium is to be determined in biological material by fluorimetry.

Table 1. Blank Signals in the MFS Procedure after Successive Purifications of the Diaminonaphthalene Reagent

<table>
<thead>
<tr>
<th>Purification method</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three extractions</td>
<td>16</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>Three extractions, and reagent dissolved in de-aerated HCl</td>
<td>14</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Three extractions, and reagent dissolved in de-aerated HCl and filtered</td>
<td>23</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

*20 μg of selenium per liter yields about 100 arb. fluorescence units.

With cyclohexane.

Table 2. Selenium Concentration (μg/g) in Lyophilized Whole Blood and Three Reference Materials

<table>
<thead>
<tr>
<th>Sample</th>
<th>MFS SD</th>
<th>HG-AAS SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilized whole blood</td>
<td>8 0.568 ± 0.026</td>
<td>0.564 ± 0.015</td>
</tr>
<tr>
<td>Serum 105</td>
<td>4 0.095 ± 0.004</td>
<td>0.088 ± 0.002</td>
</tr>
<tr>
<td>Serum 108</td>
<td>2 0.048 ± 0.002</td>
<td>0.048 ± 0.001</td>
</tr>
<tr>
<td>SRM no. 1577a</td>
<td>2 0.678 ± 0.007</td>
<td>0.695 ± 0.002</td>
</tr>
</tbody>
</table>

*Human serum, certified 90 ± 6 μg/L.

**Human urine, certified 49 μg/L.

**Bovine liver, certified 0.71 ± 0.07 μg/g.
Effect of Storage Temperature and Shaking Rate on pH and Blood-Gas Results for Two Quality-Control Products

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Directions for pre-analytical handling of ampules of two commercially available aqueous quality-control products (contrIL and G.A.S.) contain vague instructions such as "store at room temperature" and "shake vigorously" before analysis. We examined the effect of different storage temperatures (25, 31, and 36 °C) and shaking rate (one, two, and four shakes per second) on pH and blood-gas results. For both products, increasing the storage temperature significantly decreased pO2 results, the magnitude of the bias being greatest for those solutions with the highest O2 tensions. However, increasing the shaking rate partly offset this bias. Increasing storage temperature also decreased results for pCO2 and increased results for pH for both manufacturers' ampules with the highest CO2 tensions, and this bias was not offset by increasing the shaking rate. We conclude that both storage temperature and shaking rate must be precisely defined and carefully monitored before these products are used in a quality-control program.

Commercially available aqueous pH and blood-gas control products frequently are used in quality-control programs. However, the pre-analytical variables associated with handling and transferring these products to the analytical device are considered more likely to produce significant errors than are the variables intrinsic to the measurement itself (1). Unfortunately, the manufacturers of two of these products, "contrIL" and "G.A.S." blood-gas controls, give somewhat ambiguous directions regarding proper handling of the ampules before analysis. Instructions call for these materials to be stored at "room temperature" (2) and for the operator to "shake" (3) or "vigorously shake" (2) them before analysis. We noted a considerable difference among operators as to what constitutes "vigoruous" shaking, and a review of temperature records showed that "room temperature" in our laboratory was quite variable. We thus examined the effect of these two pre-analytical variables on pH and blood-gas results.

In previous reports, these variables received little attention. Komjathy et al. (4) and Abramson et al. (5) advised shaking ampules "vigorously" for 10 s before analysis; Leary et al. (6) reported shaking them for 30 s. In no case was "vigoruous" defined, nor were any data provided to support these statements. Maas et al. (7) advised keeping the ampules on the bench for a day before use and at 25 °C for 30 min before analysis. Komjathy et al. (4) stored ampules at "ambient room temperature for at least 12 h before testing." Leary et al. (6) compared pH and blood-gas results when ampules were incubated at 22 and 30 °C for 5 min before analysis and found that, at higher gas tensions, the measured pO2 decreased with increasing temperature. The potential interaction of these two pre-analytical variables, shaking rate and storage temperature, has not been examined.

Here we report the effect of storage temperature and shaking frequency on pH and blood-gas results for aqueous quality-control materials from two different manufacturers.

Materials and Methods

Materials
We purchased the blood-gas and pH quality-control materials from their manufacturers: General Diagnostics Blood G.A.S. (Gas Analyzer System) control from Organon Teknika Corp., Morris Plains, NJ 07950, and contrIL Blood Gas Control from Instrumentation Laboratory, Lexington, MA 02173. Each product consists of a set of three ampules, each ampule having different pH values and different oxygen and carbon dioxide tensions. However, the G.A.S. ampules with the lowest pH (acidosis) have the highest oxygen and lowest carbon dioxide tensions, whereas the contrIL controls with the highest oxygen and lowest carbon dioxide tensions have the highest pH (alkalosis).

Instrumentation
We made all pH and blood-gas measurements with a Model 813 Blood Gas Analyzer (Instrumentation Labora-