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Abstract

An immunoassay that detects mercuric ions in water at concentrations of 0.5 ppb and above is described. The assay utilizes a monoclonal antibody that binds specifically to mercuric ions immobilized in wells of microtiter plates. Within the range of 0.5-10 ppb mercury, the absorbance in the enzyme-linked immunosorbent assay (ELISA) is linear to the log of the mercuric ion concentration. The quantitation of mercury by ELISA correlates closely with results from cold-vapor atomic absorption. Other divalent metal cations do not interfere with the assay, although there is interference in the presence of 1 mM chloride ions. The optimum pH for mercury detection is 7.0, although 2 ppb mercury can be detected over a wide pH range. The assay is as sensitive as cold-vapor atomic absorption for mercury detection and can be performed with only 100 µl of sample.

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; EPA, Environmental Protection Agency; KLH, keyhole limpet hemocyanin; NIST, National Institute of Standards and Technology; SRM, Standard Reference Material.

Mercury and most of its derivatives are extremely toxic substances which are ubiquitous in the biosphere (1). The major source of mercury contamination is the natural degassing of the earth's crust, although major contributions also arise from anthropogenic sources (2). The amounts released from both sources do not cause problems on a global scale, but increases on a local level can lead to serious health problems with long-term consequences for the affected population (3,4). Thus, simple, sensitive, and reliable procedures for detection of mercury in the environment are needed to prevent these problems from arising.

The current method of choice for mercury analysis is cold-vapor atomic absorption spectrometry. This method has several limitations, however. First, the number of samples that can be analyzed is limited by the fact that samples are assayed one at a time. Second, a large sample volume (up to 5 ml) is often used to ensure maximum sensitivity (5), although assays performed at maximum sensitivity can lack precision (6). Third, cold-vapor atomic absorption is costly due to the use of expensive, sophisticated equipment that requires highly skilled personnel for proper operation. Finally, the method is not amenable to on-site analysis in the field.

In this paper, we describe an ELISA that detects mercury at concentrations of 0.5 ppb or greater in water. Between 0.5 and 10 ppb mercury, the absorbance is proportional to the log of the mercury concentration. The assay is specific for mercury, in that no other metal tested interferes with the quantitation of mercury. In addition, the assay requires little preliminary processing ofthe sample and can be performed with only 100 µl of sample.

Materials and Methods

Materials

Chemicals of the indicated grade were obtained from the following suppliers.

Aldrich Chemical Co.: Cadmium(II) chloride (ACS); copper(II) chloride (anhydrous, 99.999%); gold(III) chloride (99%); mercury(II) chloride (99.999%); nickel(II)

chloride (98%); selenium (IV) oxide (99.999%); and zinc(II) chloride (ACS).

Alfa Products: Silver(I) nitrate (ACS).

Baker Chemical Co.: Barium(II) chloride (ACS) and copper (II) sulfate (ACS).

EM Science: Glacial acetic acid (USP equivalent).

Fisher Scientific: Chromium (III) chloride (Certified); iron(II) sulfate (ACS); sodium acetate (ACS).

Mallinckrodt Chemical: Lead(II) acetate (AR).

Sigma Chemical: Mercury(II) chloride.

An EP extract metals quality control sample was obtained from the Environmental Protection Agency, Quality Assurance Branch, Environmental Monitoring and Support Laboratory (Cincinnati, OH). The sample contained 0.2 mg/liter Hg^{2+}, 100 mg/liter Ba^{2+}, 1 mg/liter Cd^{2+}, 5 mg/liter Cr^{3+}, 5 mg/liter Pb^{2+}, and 5 mg/liter Ag^{+} in distilled water adjusted to pH 5.0 with acetic acid.

Standard Reference Materials 1641 and 3133 were obtained from the National Institute of Standards and Technology, Office of Reference Materials (Gaithersburg, MD). SRM 1641 consisted of mercury at a concentration of 1.52 μg/ml in 2% nitric acid, and SRM 3133 contained 10 mg/ml mercury (as 16.2 mg/ml mercuric nitrate) in 10% nitric acid.

Mercury-Specific Hybridoma Antibodies

Monoclonal antibodies were produced by injection of BALB/c mice with glutathione-$HgCl_2$ conjugated to keyhole limpet hemocyanin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (7). Antibodies were screened for reactivity with BSA-glutathione and BSA-glutathione-$HgCl_2$. Those which reacted with only BSA-glutathione-$HgCl_2$ were presumed to be specific for mercuric ions. Two antibodies which satisfied this criterion were isolated. A more complete description of production and characterization of these antibodies will be presented elsewhere (D. Wylie *et al.,* manuscript in preparation).

Detection of Mercury in Water by Enzyme-Linked Immunosorbent Assay

Ninety-six-well microtiter plates (EIA/RIA grade, Costar Corp., Cambridge, MA) were treated with BSA-glutathione, blocked, and used for the ELISA. One hundred-microliter aliquots of water containing known amounts of mercuric chloride, ranging from 0.2 to 200 ppb, were added to the wells of the microtiter plate for 30 min. The plates were washed three times, and then ascites fluid containing a mercury-specific monoclonal antibody was added for 30 min at room temperature, followed by goat anti-mouse μ chain conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). After incubation for 30 min at room temperature, the plates were washed, and 100 μl of ABTS peroxidase substrate (Kirkegaard and Perry Laboratories) was added to each well. After 15 min of incubation, the absorbance of each well at 405 nm was measured with a Titertek Multiscan MC multichannel spectrophotometer (Flow Laboratories, Rockville, MD).

Cold-Vapor Atomic Absorption Spectrometry

Mercury concentrations of some samples were determined by cold-vapor atomic absorption in the Diagnostic Laboratory, Department of Veterinary Sciences, University of Nebraska–Lincoln with a Mercury Monitor flame less atomic absorption spectrophotometer (Model 1255, Milton Roy Inc., LDC Division, Riviera Beach, FL). Before analysis, the samples were treated with $SnCl_2$ in 10% HCl to reduce mercuric ions to elemental mercury, and the mercury concentration was determined by comparison with a $HgCl_2$ standard (mercury reference standard solution, Fisher Scientific) treated in the same manner.

For direct comparison of mercury quantitation by atomic absorption and ELISA, the mercury standard was diluted to a nominal concentration of 100 ppb in 0.1 M Hepes, pH 6.8. Two aliquots were then removed. One was diluted in Hepes buffer to the appropriate concentrations for analysis by immunoassay, while the other was diluted in 10% nitric acid ("Baker analyzed" 70- 71%, Trace Mineral Analysis, Baker Chemical Co.) for atomic absorption measurement. Mercury was then measured as described above for each method.

Mercury Quantitation in EPA and NIST Samples

Each of the samples from the Environmental Protection Agency and the National Institute of Standards and Technology was diluted in water to mercury concentrations of 1-200 ppb and then used in the ELISA as described above. The results were compared with a standard curve constructed from ELISA analysis of water containing known concentrations of mercury. The mercury concentrations of the samples used for construction of the standard curve were also measured by atomic absorption.

Interference with Mercury Detection by Other Metals in the ELISA

A 2 mM solution of each metal salt in water was diluted to concentrations of 20 μM, 200 nM, 20 nM, and 2 nM. Fifty microliters of each concentration was added to individual microtiter wells treated with BSA-glutathione. Fifty microliters of SRM 3133 containing mercury at concentrations ranging from 1 to 200 ppb was added to the appropriate wells. The plates were incubated at room temperature for 30 min, after which the plates were washed and assayed by the ELISA described above.

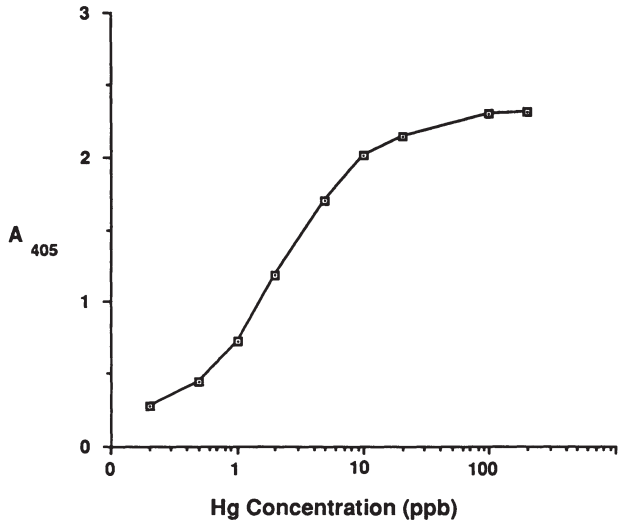

Figure 1. Detection of mercury in water by Hg^{2+}-specific ELISA. The ELISA described under Materials and Methods was used to determine the absorbance at 405 nm obtained with known concentrations of mercuric chloride in 0.1-ml aliquots of water. Each point represents the average absorbance obtained from seven separate analyses of each mercuric chloride concentration. Background absorbance obtained by analysis of an aliquot of water without added mercuric chloride was 0.137.

pH Dependence of Mercury Detection by ELISA

A solution of 10 mM sodium acetate was adjusted to the desired pH by addition of either 1 N HCl or 1 N NaOH. SRM 3133 was added to these solutions to give a final mercury concentration of 2 ppb. The pH of the samples was measured again at this point to ensure that it remained at the desired value. One hundred microliters of each solution was added to BSA-glutathione-treated microtiter plates and assayed by ELISA. Controls consisting of 10 mM sodium acetate at the appropriate pH value and of known concentrations of SRM 3133 in distilled water were included in each assay.

Hazards and Precautions

Since mercuric chloride and several ofthe other metal salts used are toxic, rubber gloves were worn to prevent contact with the skin. Also, exposure to dust was kept to a minimum. All work with samples that might contain metallic mercury was performed in a fume hood.

Results and Discussion

An immunoassay capable of detecting small amounts of mercury in water was developed with the use of an antibody that reacts with immobilized mercuric ions. The production and characterization of the antibody, which was induced by injection of BALB/c mice with glutathione-$HgC1_2$ conjugated to KLH, will be described elsewhere (D. Wylie *et at.,* manuscript in preparation). The results obtained when various concentrations of mercuric chloride in water were assayed by the ELISA procedure described under Materials and Methods are shown in Figure 1. Absorbance that was approximately twice that of background was consistently noted for mercuric ion concentrations as low as 0.5 ppb when compared to water with no added mercury, and concentrations of 0.2 ppb were 50% above background. Frequently, concentrations of mercuric ions at 0.1 ppb demonstrated absorbance in this same range. A linear relationship between A_{405} and the log of the mercury concentration was obtained in the range of 0.5-10 ppb, as indicated by a correlation coefficient of 0.998 within this interval.

In addition to its linearity, the assay was highly reproducible. Table 1 shows the results of seven replicates for each mercury concentration, along with the means, standard deviations, and coefficients of variation. Standard deviations were less than 11% of the mean at 0.2 ppb and generally decreased as the concentration of Hg^{2+} increased to 10 ppb. In all cases, except for the sample containing no Hg^{2+}, the coefficient of variation was 10% or less, again reflecting the reproducibility of the assay. These results also indicated that the ELISA was as sensitive for mercuric ion detection as the atomic absorption procedure recommended by the EPA, which is also capable of mercury detection down to 0.2 ppb, but requires a 100-ml sample to do so (8).

Although Figure 1 clearly shows that the ELISA was capable of sensitive and reproducible detection of mercuric ions in water, it did not reveal how well the

Table 1. Statistical Analysis of ELISA Data from Mercury Detection in Water

	Mercury concentration (ppb)						
Replicate	0.0	0.2	0.5	1.0	2.0	5.0	10.0
1	0.196[a]	0.302	0.401	0.759	1.123	1.592	2.064
2	0.153	0.272	0.469	0.765	1.180	1.750	2.000
3	0.140	0.272	0.413	0.749	1.338	1.665	1.988
4	0.123	0.278	0.496	0.787	1.323	1.817	2.053
5	0.108	0.237	0.445	0.711	1.195	1.751	1.963
6	0.123	0.303	0.398	0.716	1.093	1.610	2.059
7	0.113	0.280	0.520	0.588	1.044	1.717	1.968
Mean	0.137	0.278	0.449	0.725	1.185	1.700	2.014
SD	0.030	0.022	0.048	0.066	0.112	0.082	0.044
Coeff. var.	22.277	7.994	10.710	9.118	9.419	4.806	2.187

a. Values represent the absorbance at 405 nm of ELISA analyses performed as described under Materials and Methods. The data shown are the same as those used to derive the graph in Figure 1. The correlation coefficeint *(r)* between A_{405} and the log of the mercury concentration between 0.2 and 10 ppb is 0.998.

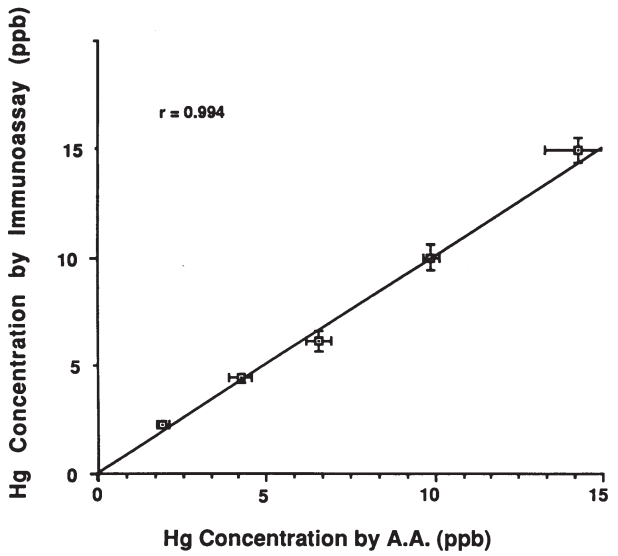

Figure 2. Comparison of mercury detection by ELISA and atomic absorption, (A.A.). An atomic absorption mercury reference standard was diluted to mercury concentrations of 2, 4, 6, 10, and 15 ppb either in 0.1 M Hepes, pH 6.8, for analysis by ELISA or in 10% nitric acid for cold-vapor atomic absorption. Each sample was then analyzed as described under Materials and Methods. The values shown represent the mean and one standard deviation of quadruplicate analyses by immunoassay and triplicate analyses by atomic absorption.

results correlated with atomic absorption analysis. This is an important consideration, since cold-vapor atomic absorption is currently the method of choice for mercury determination. To correlate the results obtained from the two methods, an atomic absorption mercury reference standard was diluted in 0.1 M Hepes, pH 6.8, to a mercury concentration of 100 ppb. At this point, two aliquots were removed and diluted to the appropriate concentrations for immunoassay or atomic absorption as described under Materials and Methods. Samples containing 0, 2, 4, 6, 10, and 15 ppb mercury were then analyzed by both methods. As shown in Figure 2, the results obtained from the two methods were in close agreement, as indicated by a correlation coefficient of >0.99. In addition, the standard deviation of the immunoassay at most mercury concentrations was the same or less than that obtained by atomic absorption. These results demonstrated that, under the conditions of this assay, quantitation of mercury by ELISA was as precise as that by cold-vapor atomic absorption.

The specificity of the assay for mercury was investigated with the use of an EPA quality control sample containing 0.2 mg/liter Hg^{2+}, 100 mg/liter Ba^{2+}, 1 mg/liter Cd^{2+}, 5 mg/liter Cr^{3+}, 5 mg/liter Pb^{2+}, and 5 mg/liter Ag^{+} in distilled water adjusted to pH 5.0 with acetic acid. The sample was diluted to known Hg^{2+} concentrations, which were assayed by ELISA and compared to results obtained with standards consisting of known concentrations of mercuric chloride in water (Figure 3). Significant reactivity was obtained with both the EPA sample and the water standard at 0.2 ppb mercury, and the absorbance for both samples was linear up to 20 ppb mercury. Reactivity was due to the presence of mercury and not to recognition of one of the other metals, since a sample containing all of the metals except mercury in the same concentrations as in the EPA sample gave the same absorbance as water containing no mercury.

The results in Figure 3 indicated that the metals in the EPA sample did not interfere with mercury detection at the concentrations at which they were present when the sample was diluted to give mercury concentrations between 0.2 and 200 ppb. The results, however, did not reveal whether higher concentrations of these or other metals would interfere with the assay. Therefore, concentrations of individual metal ions from 1 mM to 10 nM were examined for interference with detection of various concentrations of mercury in SRM 3133 (Figure 4). Several metal salts, including ferrous sulfate, lead acetate, selenium dioxide, and silver nitrate, did not inhibit mercury detection, even when they were present at a concentration of 1 mM and mercuric ion was only 2 ppb. The results shown in Figure 4a were obtained when lead acetate was analyzed for interference and are representative of those obtained with the other metals mentioned above.

Several other metal salts, however, including barium chloride, cadmium chloride, chromic chloride,

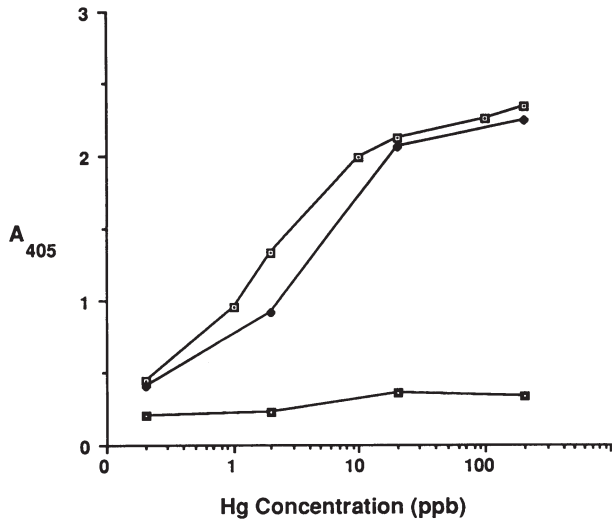

Figure 3. Detection of mercury in the EPA quality control sample by ELISA. The QC sample (♦) and $HgCl_2$ (□) were diluted in water to mercury concentrations ranging from 0.5 to 200 ppb and then analyzed by ELISA as described under Materials and Methods. A sample containing the same concentration of all other metals as the QC sample except mercury was also included (■). The absorbance obtained in analysis of both water without added mercury and the EPA sample without mercury was 0.263. Each point represents the average absorbance obtained from quadruplicate analyses of each sample.

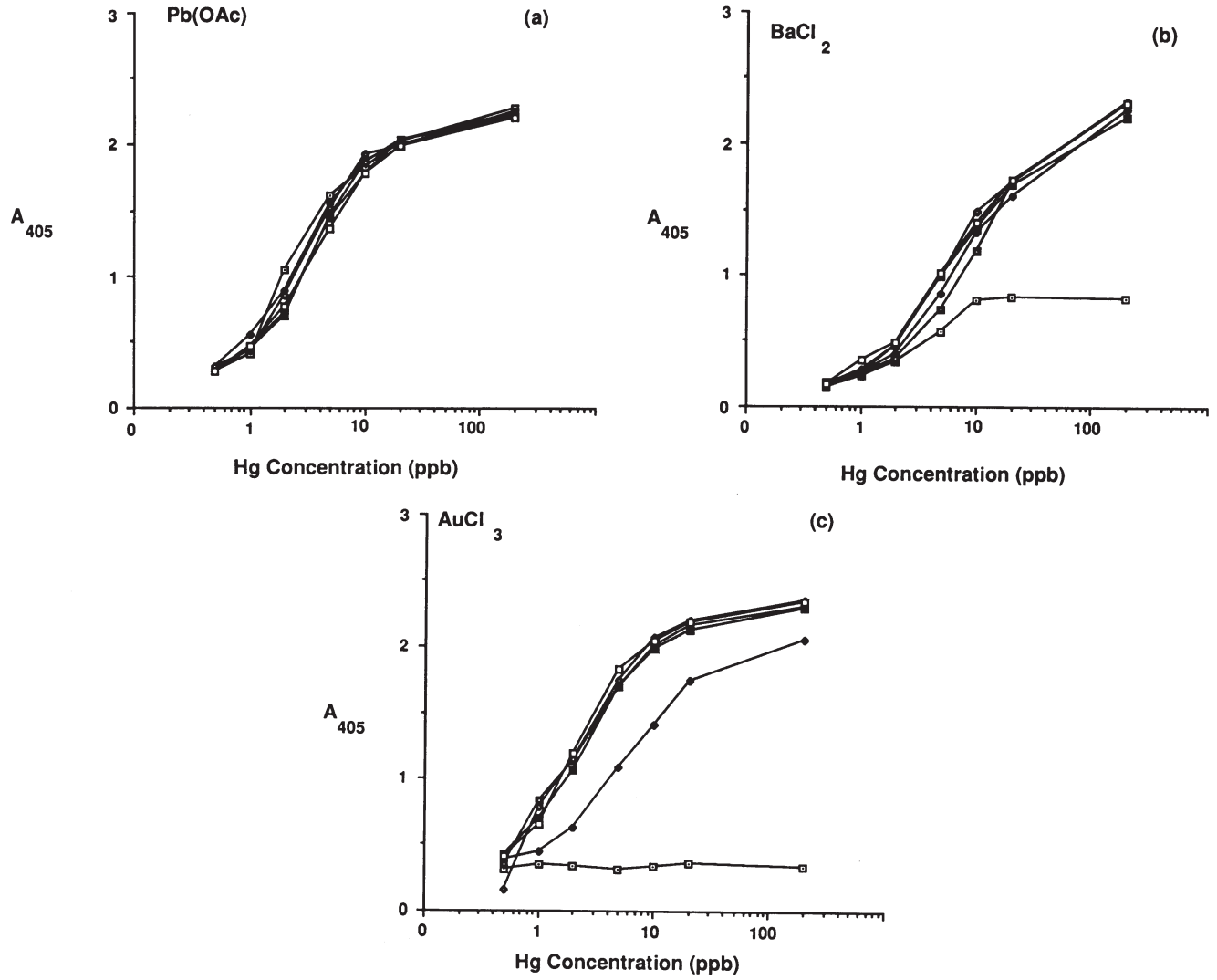

Figure 4. Effect of metal salts on mercuric ion detection by ELISA. Various metal salts at 0 nM (□), 1 nM (■), 10 nM (◊),100 nM (⧈), 10 μM (♦), and 1 mM (⊡) concentrations were added to mercuric ion standards to determine their effects on the quantitation of mercury at concentrations ranging from 0.5 to 200 ppb. For each concentration of metal salt, a control containing the same concentration of metal salt but with no added mercury was included. These values were below 0.2. Each point represents the average absorbance obtained from quadruplicate analyses of each sample. The figures are labeled with the chemical symbol for the inhibiting salt.

cupric chloride, gold chloride, nickel chloride, and zinc chloride, did interfere, but usually only at the highest concentration (1 mM), although gold chloride also demonstrated interference at 10 μM. Figure 4b represents results obtained with barium chloride, which is typical of all metal chloride salts tested, except gold chloride, which also demonstrated some interference at a concentration of 10 μM (Figure 4c).

Interestingly, all the metals that interfered in the assay were used as the chloride salt, suggesting that the chloride ion, rather than the metal cation, was responsible for the interference. This was tested directly by comparison of the sensitivity of the assay in the presence of $CuCl_2$, $CuSO_4$, and NaCl (Figure 5). It can be seen that $CuSO_4$ did not interfere with the assay at any concentration, whereas $CuCl_2$ and NaCl did at 1 mM, but not at 10 μM. These data clearly establish that the inhibition is due to chloride ions. Thus, the metal cations tested do not interfere with the quantitative detection of mercuric ions.

Interference by chloride ions was most apparent at concentrations of mercury above 1-2 ppb, except in the case of gold chloride. This could pose technical problems for analysis of samples, such as seawater, which contain chloride in concentrations that might interfere. However, dilution of the samples to lower the chloride concentration or dilution of both the test samples and the samples used for construction of the standard curve in chloride-containing buffer might allow the ELISA procedure to be used in these situations.

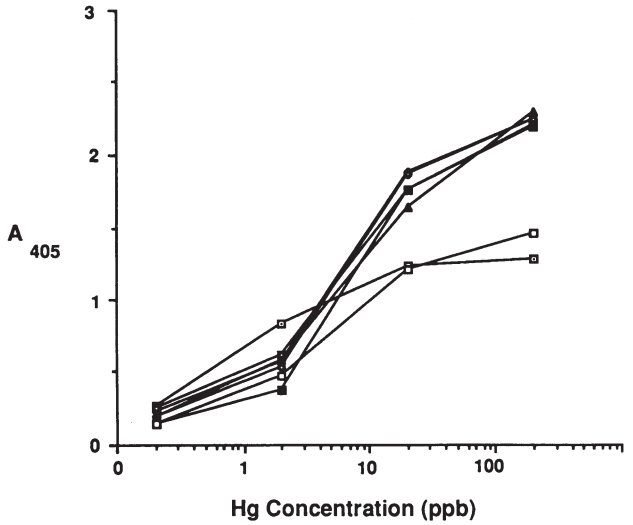

Figure 5. Effect of chloride ions on mercuric ion detection by ELISA. Mercuric ion standards from 0.5 to 200 ppb were measured by ELISA in the presence of 1 mM (⊡) and 10 μM $CuCl_2$ (♦), 1 mM (◘) and 10 μM $CuSO_4$ (◊), and 1 mM (■) and 10 μM NaCl (□). Mercuric ion standards without added metal salts (A) were included as controls. For each concentration of metal salt, a control containing the same concentration of metal salt but with no added mercury was included. The absorbance values of these samples were 0.230 or less. Each point represents the average absorbance obtained from quadruplicate analyses of each sample.

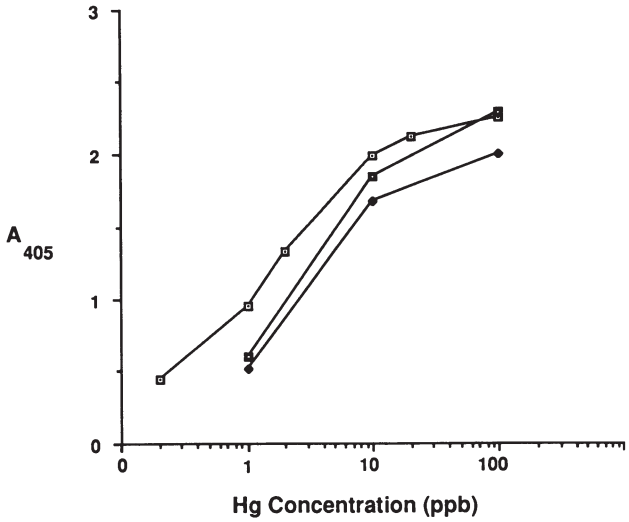

Figure 6. Detection of mercury in National Institute of Standards and Technology Standard Reference Materials. SRM 1641 (♦) and SRM 3133 (◘) were diluted in water to mercury concentrations of 1, 10, and 100 ppb and then analyzed by ELISA as described under Materials and Methods. A control consisting of known concentrations of mercury in water was included for comparison (⊡). SRM 1641 consisted of metallic mercury at a concentration of 1.52 μg/ml in 2% nitric acid, and SRM 3133 contained 16.2 mg/ml mercuric nitrate in 10% nitric acid. Each point represents the average absorbance obtained from quadruplicate analyses of each sample.

The results presented thus far indicate that the immunoassay is as sensitive as conventional cold-vapor atomic absorption for mercuric ion detection and requires 100-fold less sample for analysis (100 μl versus up to 5 ml for detection of 1 ppb mercury by atomic absorption) (5). However, since most samples for mercury analysis by cold-vapor atomic absorption are stabilized by addition of nitric acid (9), it was of interest to determine whether the immunoassay could detect mercury in samples treated similarly. Two samples obtained from the National Institutes of Standards and Technology, SRM 3133, which consisted of mercuric acetate in 10% nitric acid, and SRM 1641, which contained metallic mercury in 2% nitric acid, were assayed by the Hg^{2+}-specific ELISA. Each sample was diluted to mercury concentrations from 1 to 100 ppb before analysis. As shown in Figure 6, mercury could be detected in each sample at concentrations of 1 ppb, although the absorbance at that concentration was approximately half that obtained with water containing the same amount of mercury. One potentially interesting implication from these results is that the ELISA could detect mercury in SRM 1641, which contained elemental mercury, indicating that the immunoassay might also be capable of detecting this form of mercury under the conditions of the assay.

In the experiments shown in Figure 6, when the SRM samples were diluted to the appropriate mercury concentrations, the acid in which the solutions had been stored was presumably diluted sufficiently so it would not interfere with the assay. However, samples containing unknown mercury concentrations must be analyzed at several dilutions to determine the one appropriate for quantitation by ELISA. The pH of each dilution would depend upon its acid concentration. Therefore, it was important to determine the effect of pH on the initial step of mercury immobilization, since all subsequent steps in the ELISA are performed after the sample has been removed from the ELISA plate and it has been washed with buffered saline. To do this, SRM 3133 was diluted in 10 mM sodium acetate to a mercury concentration of 2 ppb. This solution was then adjusted to the desired pH by addition of either 1 N HCl or 1 N NaOH. Each sample was subsequently analyzed by ELISA for the presence of mercury (Figure 7). The results show that, even though mercury could be detected over a wide pH range, the assay was most sensitive from pH 6 to 10, with an optimum at 7.0. The decrease in sensitivity of the assay was more pronounced at acid, rather than alkaline, pH, with the absorbance at pH 10 approximately the same as that at pH 6.

The potential problems posed by the effect of pH on the ELISA could be circumvented by addition of a suitable buffer to raise the pH of samples that have been acidified for atomic absorption analysis. The feasibility

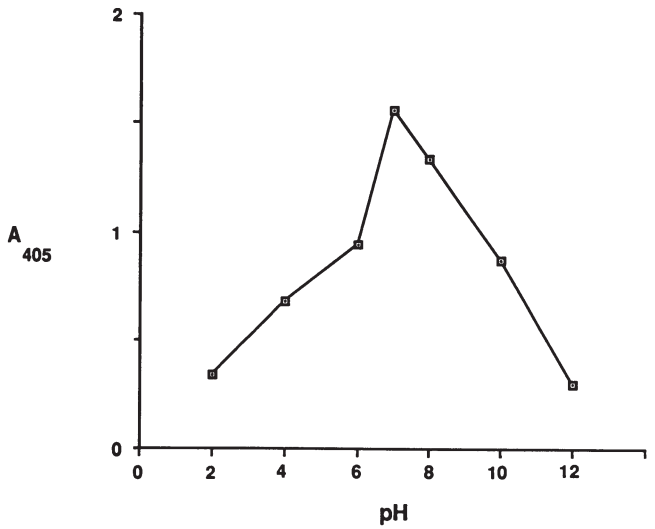

Figure 7. Effect of pH on mercury detection by ELISA. Solutions containing 2 ppb mercury in 10 mM sodium acetate were adjusted to the appropriate pH with either 1 N HCl or 1 N NaOH. The solutions were then used in the ELISA as described under Materials and Methods. For each pH value, the absorbance of sodium acetate containing no added mercury was also determined and found to be similar to that of water without added mercury. The absorbance values for these samples were below 0.2. Each point represents the average absorbance obtained from quadruplicate analyses of each sample.

of this approach is demonstrated by the results in Figure 2, in which an atomic absorption standard with 100 ppb mercury in 10% nitric acid was diluted in Hepes buffer for ELISA analysis and in 10% nitric acid for atomic absorption analysis. The results of mercury quantitation obtained with the two methods were very similar, with a correlation coefficient > 0.99.

ELISA assays have been adapted to a variety of analytical procedures in recent years because of the exquisite specificity of monoclonal antibodies. The assay described here involves recognition of immobilized mercuric ions by a Hg^{2+} specific monoclonal antibody. Although metal ions have been reported to influence the reactivity of antibodies with their specific epitopes (10-15), the antibody used for this assay is unique in that it binds to mercuric ions attached to a glutathione-protein carrier but will not recognize the carrier without mercury present. Further investigation has revealed that soluble mercuric ions will bind to the antibody (D. Wylie *et al.,* manuscript in preparation).

The use of an ELISA for detection of metal ions circumvents many problems associated with atomic absorption. For instance, samples can be analyzed in parallel, enabling large numbers of samples to be processed at one time. In addition, quantitative analysis can be performed with a simple spectrophotometer or microtiter plate reader. Automation of the photometer thus makes practical the processing of a large number of samples, allowing for the implementation of large-scale monitoring programs. Since the assay yields a visible color change, semiquantitative procedures which require no electronic instrumentation for evaluation can be developed. Thus, the assay has the potential for field use. Finally, the procedure requires only 0.1 ml of sample, up to 1000-fold less than that required by atomic absorption for maximum sensitivity, and it can, therefore, be used to analyze samples available in volumes insufficient for cold-vapor atomic absorption.

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