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Detection of Heavy Metals by Immunoassay: Optimization and Validation of a Rapid, Portable Assay for Ionic Cadmium

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An immunoassay is described that measured Cd(II) in aqueous samples at concentrations from approximately 7 to 500 ppb. The assay utilized a monoclonal antibody that bound tightly to a cadmium−ethylenediaminetetraacetic acid (EDTA) complex but not to metal-free EDTA. A inhibition immunoassay format was employed for this analysis; ionic cadmium was diluted into an excess of EDTA before being incubated with the antibody in the presence of an immobilized Cd(II)−EDTA conjugate. Ca(II), Na(I), and K(I), cations commonly encountered in ambient water samples, did not interfere with the cadmium immunoassay at concentrations approaching their solubility limit. The assay reliably measured Cd(II) in the presence of a 1 mM excess of Fe(III), Mg(II), and Pb(II). Zn(II) and Ni(II) had minimal effect on the assay at levels below 100 μM, and the immunoassay was relatively insensitive to interferences by In(III) and Mn(II) at concentrations up to 10 μM. Hg-(II) had the ability to cause a false positive in the assay, but only at concentrations higher than 1 μM. The assay compared favorably with atomic absorption spectroscopy in its ability to measure cadmium in spiked water samples taken from a Louisiana bayou.

INTRODUCTION

Cadmium is an environmental toxin that accumulates in the body and has a biological half-life of greater than 10 years in humans (*1*). Depending upon the route of ingestion, high levels of cadmium exposure can affect kidney or lung function (*2*). In addition, experimental and epidemiologic studies are providing substantial evidence that low-level chronic exposure, in combination with other environmental factors, can contribute to an increased risk of cancer (*3*). Thus, the ability to rapidly and inexpensively monitor environmental cadmium is a prerequisite for effecting USEPA-mandated reductions in contaminant loadings to minimize human and animal exposure. According to current USEPA regulations, drinking water can contain no more than 4 ppb dissolved cadmium (*4*). Criteria (which vary depending on water hardness) have also been established for maximum dissolved cadmium loadings for acute and chronic exposure in fresh and marine waters (*4*). For example, the acute and chronic exposure criteria for dissolved cadmium in fresh water are 3.7 and 1.0 ppb, respectively (at water hardness of 100 mg/L as $CaCO_3$); in marine waters, the acute and chronic cadmium exposure criteria are 9.3 and 4 ppb, respectively. Currently, environmental samples are analyzed by either graphite furnace atomic absorption spectroscopy (GFAAS) (*5*) or inductively coupled plasma spectroscopy (ICPAES and ICPMS) (*6*). The purpose of this study was to develop an antibody-based assay for cadmium that would provide an adjunct to more traditional methods of analysis.

Immunoassays are becoming increasingly accepted for environmental applications. These assays are quick, inexpensive, simple to perform, and sufficiently portable to be used at the site where the sample is taken. Although most environmental immunoassays are directed toward low molecular weight organic compounds, including industrial pollutants, pesticides, and herbicides (*7*−*9*), the technique is theoretically applicable to any pollutant for which a specific antibody can be generated. An antibody-based immunoassay for ionic mercury described in 1991 is the basis for the only metal ion immunoassay presently available commercially (*10*). This assay captured soluble ionic mercury on a reactive sulfhydryl surface and utilized a mercury-specific monoclonal antibody to bind the mercury−sulfhydryl complex. Our laboratory has previously described a prototype immunoassay for the measurement of chelated complexes of heavy metals in environmental samples (*11*). This assay utilized an antibody that recognized EDTA complexes of indium and measured indium concentrations from 0.02 ppb to 300 ppm.

In this study, we report features of an immunoassay that measures the levels of cadmium contamination in environmental samples. This immunoassay used a newly described monoclonal antibody that recognized cadmium−EDTA complexes but not metal-free EDTA (*12*) and employed a format very similar to that described previously for the prototype heavy metal immunoassay (*11*).

EXPERIMENTAL SECTION

Chemicals. Bovine serum albumin (fatty acid ultrafree) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). 1-(4-Isothiocyanobenzyl)ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid was purchased from Dojindo Laboratories (Kumamoto, Japan). Cadmium foil (99.999%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Atomic spectroscopy standard cadmium, indium, magnesium, manganese, zinc, lead, iron, nickel, and mercury (1000 ppm in 1 N HNO_3) were obtained from Perkin-Elmer Corporation (Norwalk, CT). Goat anti-mouse IgG (Fc specific) conjugated to horseradish peroxidase was purchased from Sigma Chemical Co. (St. Louis, MO). ELISA microwell plates were a product of Costar, Inc. (Cambridge, MA). All water was purified by filtration through a Nanopure II water purification system (Barnstead/Thermolyne, Dubuque, IA). Metal-free disposable pipet tips were a product of Oxford Labware, Inc. (St. Louis, MO). All glassware was mixed-acid washed (*13*) and liberally rinsed with purified water, and all plasticware was soaked overnight in 3 M HCl and rinsed liberally with purified water before use.

Antibody and Cd(II)-EDTA-BSA Conjugates. The isolation and characterization of the monoclonal antibody

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synthesized by the hybridoma cell line 2A81G5 has been previously described by our laboratory (*12*). To prepare antibody for these studies, the hybridoma cell line was grown as an ascites in BALB/c mice primed with Freund's incomplete adjuvant (*14*). The monoclonal antibody was purified by affinity chromatography on a Affigel Protein G column (Pierce Chemical Co., Rockford, IL), as described by the manufacturer. The bovine serum albumin-thioureido-L-benzylethylenediaminetetraacetic acid−cadmium conjugate [Cd(II)−EDTA-BSA] was prepared and characterized as previously described (*12*). The conjugate used in these studies had EDTA moieties on approximately 15% of the lysine residues.

Optimization of Reagent Concentrations and Inhibition ELISA. The optimum conjugate concentration for coating microwell plates and the best working dilutions for the purified 2A81G5 monoclonal antibody were determined by ELISA. Cd(II)−EDTA-BSA conjugate was diluted into PBS at concentrations of 0.5, 1.0, and 2.0 μg/mL and adsorbed to ELISA microwell plates overnight at 4 °C. The plates were washed with 0.05% Tween 20 in 137 mM NaCl, 3 mM KCl, and 10 mM sodium phosphate buffer, pH 7.4 (PBS), and wells were blocked with 3% BSA in PBS. The 2A81G5 monoclonal antibody was serially diluted through the wells of the microwell plate, and the antibody was allowed to incubate in the plate for 1 h at 25 °C. Goat anti-mouse IgG-horseradish peroxidase conjugate and TMB Microwell Substrate (Kirkegaard-Perry Laboratories, Gaithersburg, MD) were used for color development. The absorbance of each well was measured in the dual wavelength mode (450−650 nm) using a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA), and the data was transformed to a four-parameter curve using SoftMax software provided with the instrument. For competitive ELISA, soluble antigens were preincubated with purified 2A81G5 monoclonal antibody (0.1 μg of IgG/mL in PBS + 1% BSA) for 1 h at room temperature, and then a 50 μL aliquot of this solution was added to each well of a 96-well microtiter plate coated with 0.5 μg/mL Cd−EDTA-BSA conjugate. EDTA was used at a concentration of 115 mM during the preincubation of the soluble metal with the monoclonal antibody. Because EDTA has a very high affinity for metals, any soluble metal present in the assay mixture will exist as a metal−EDTA complex. Soluble inhibiting antigens used in different experiments included the following: In(III)−EDTA, Hg(II)−EDTA, Mn(II)−EDTA, Fe(III)−EDTA, Pb(II)−EDTA, Mg(II)−EDTA, Ni(II)−EDTA, and Zn(II)−EDTA.

Collection and Preparation of Environmental Samples. Environmental water samples were collected in Bayou Trepagnier, located approximately 22 miles west of Metropolitan New Orleans adjacent to the Bonnet Carré Spillway. Over the past 80 years, the bayou has received cooling water, process waste water, and surface runoff from the Shell Norco Manufacturing Complex; its bottom sediments and soils are heavily polluted with heavy metals (Pb, Zn, and Cr) and with a variety of polycyclic aromatic hydrocarbons (*15*, *16*). Bayou Trepagnier was chosen to test the cadmium immunoassay because its water chemistry is typical of polluted bayous found in southern Louisiana. Water samples from the bayou were collected in precleaned gallon jugs and transported back to the laboratory on ice. Water used in making spiked samples for the immunoassay was first filtered through a Whatman 43 filter to remove coarse particulates. The filtered water was then passed through a 0.45 μm syringe filter and stored in precleaned 50 mL centrifuge tubes. A series of cadmium-spiked samples were made in the concentration range 40−4000 ppb by diluting a cadmium standard (Spex, 10 000 ppm Cd(II) in 5% nitric acid) with bayou water. Additional test solutions were made using deionized water from a Modupure Plus reagent grade water system. The dissolved cadmium content of the test solutions were confirmed prior to immunoassay using a Perkin-Elmer 4100ZL graphite furnace atomic absorption spectrophotometer. All test solutions were found to be within 5% of the calculated value.

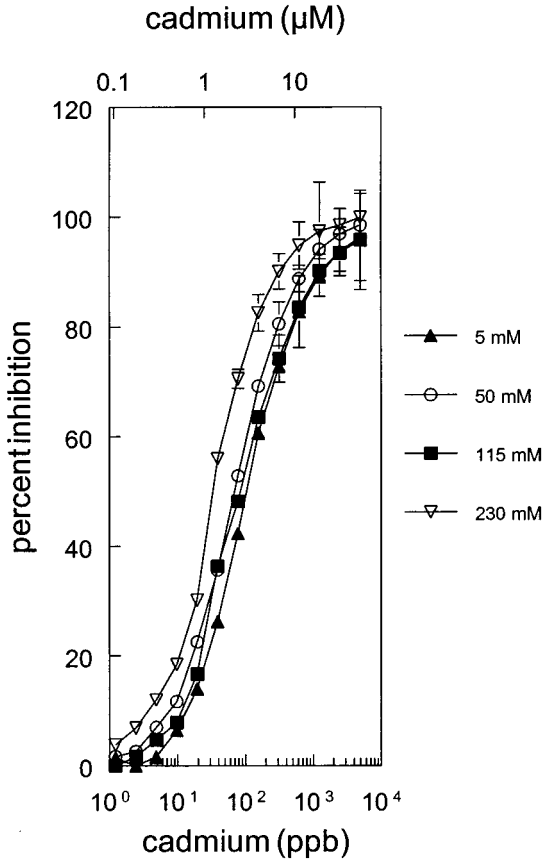

FIGURE 1. Effect of EDTA concentration on assay sensitivity. Competitive ELISAs were performed using atomic absorption grade cadmium diluted into varying EDTA concentrations as shown: (▲) 5 mM; (○) 50 mM; (■) 115 mM; (▽) 230 mM. Each point represents the mean of three determinations ± SD.

Analysis of Environmental Water Samples for Cadmium. The pH of the water samples was adjusted to 7.2 by the addition of a 10% volume of a concentrated buffer solution containing 1.37 M NaCl, 30 mM KCL, and 100 mM HEPES, pH 7.4. Samples were subsequently diluted into HEPES-buffered saline (HBS, 137 mM NaCl, 3 mM KCl, and 10 mM HEPES, pH 7.4) to bring them into the linear range of the immunoassay. A 50 μL aliquot of each diluted sample was mixed with 100 μL of 2A81G5 antibody solution (0.15 μg/mL of purified antibody in HBS containing 115 mM EDTA and 0.75% BSA), and the duplicate 50 μL aliquots of the mixture were added to wells of a microwell plate coated with 0.5 μg/mL of Cd−EDTA-BSA and blocked with 3% BSA. After 1 h at 25 °C, the plates were washed, and the amount of bound 2A81G5 antibody was quantified using goat anti-mouse IgG-horseradish peroxidase conjugate and TMB Microwell Substrate as described above. Standard curves for cadmium were obtained using the same procedure on plates of the same series. The data was analyzed by nonlinear regression (Enzfitter, Biosoft) to estimate the parameters of an empirical fitting equation:

$$OD = OD_{max} - Cap[Cd(II)]/(IC_{50}+[Cd(II)])$$

where OD_{max} is optical density signal in the absence of soluble Cd(II), OD is the optical density in the presence of a known quantity of soluble Cd(II), Cap is the maximal decrease in

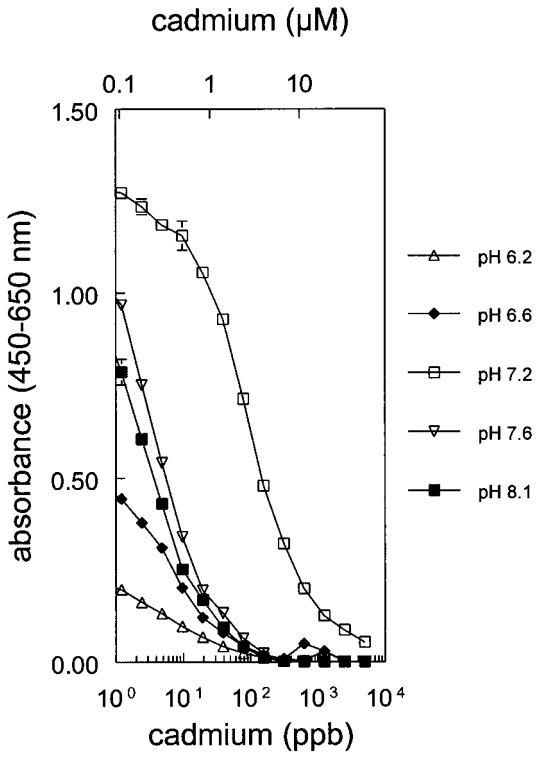

FIGURE 2. Effect of pH on assay sensitivity. Competitive ELISAs were performed using atomic absorption grade cadmium diluted into 115 mM EDTA at the pHs indicated: (△) pH 6.2; (◆) pH 6.6; (□) pH 7.2; (▽) pH 7.6; (■) pH 8.1. Each point represents the mean of three determinations ± SD.

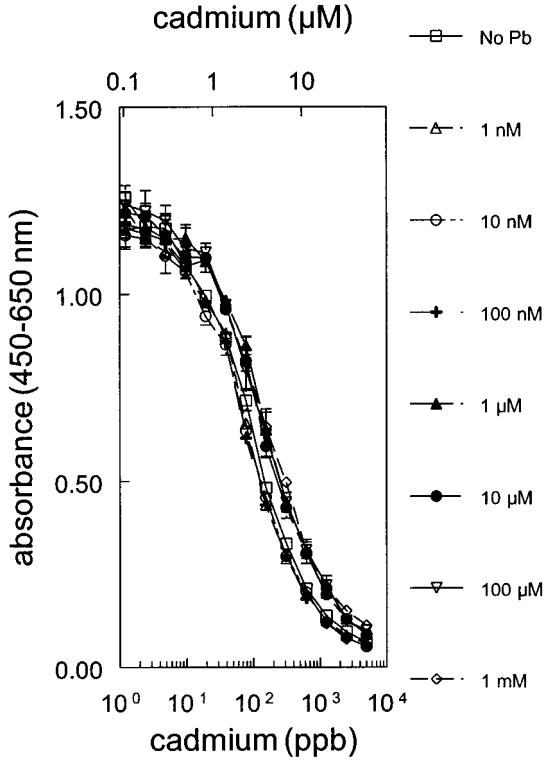

FIGURE 3. The effect of Pb(II) on the cadmium immunoassay. Competitive ELISAs were performed in the absence of added Pb (□), or in the presence of increasing Pb(II) concentrations as shown: (△) 1 nM; (○) 10 nM; (+) 100 nM; (▲) 1 μM; (●) 1 μM; (▽) 100 μM; (◇) 1 mM. Each point represents the mean of three determinations ± SD.

the OD at saturating Cd(II) concentrations, and IC_{50} is the cadmium concentration that produces a 50% inhibition in the signal.

The concentrations of cadmium in the environmental samples (X) were then calculated as weighted averages for 2 to 4 measurements at appropriate dilutions as

$$X = IC_{50}Id/(Cap/(OD_{max} - OD) - 1)$$

where Id is a dilution of the sample. The weighting function was derived according to the general rules (*17*) from standard errors in parameters of the calibration curves and standard errors at subsequent dilutions.

RESULTS AND DISCUSSION

Effect of Coating Reagent, EDTA Concentration, and pH on Assay Performance. The purified monoclonal antibody produced by hybridoma 2A81G5 bound tightly to Cd(II)−EDTA-BSA conjugate coated onto 96-well plates; the competitive immunoassay was most sensitive when the conjugate was coated at a concentration of 0.5 μg/mL (data not shown), and this concentration of coating reagent was used in all subsequent experiments. The assay was relatively insensitive to metal-free EDTA over a wide range of concentrations, as shown in Figure 1. The response to cadmium in a competitive immunoassay was virtually identical at EDTA concentrations from 5 to 115 mM; unless otherwise noted, 115 mM EDTA was used as the standard EDTA concentration for all subsequent immunoassays. This high concentration of EDTA ensures that any metal cation in the immunoassay would be present as a metal−EDTA complex, the form of the metal recognized by the monoclonal antibody. The effect of pH on the immunoassay is shown in Figure 2. The assay response was very dependent upon the pH of the incubation mixture; response was optimal between pH 7.0 and 7.2, but was strongly inhibited when the pH of the incubation mixture fell below 7.0 or above 7.3. In a competitive immunoassay, any factor which depresses color formation will be read as a positive response; the strict pH dependence displayed by the 2A81G5 monoclonal antibody requires that the pH of the incubation mixture be carefully controlled to avoid false positives when assessing environmental samples. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.0 was chosen as the buffer for subsequent environmental analyses because of its pK_a and its negligible metal-binding capacity (*18*). The pH of all environmental samples was adjusted with HEPES and checked before the samples were subjected to analysis by the immunoassay.

Effect of Other Metal Ions on the Cadmium Immunoassay. The 2A81G5 monoclonal antibody has been shown to interact with metal−EDTA complexes with equilibrium dissociation constants (K_ds) ranging from 21 nM for Cd(II)−EDTA to 820 μM for Al(III)−EDTA (*12*). Because metals are ubiquitous in the environment, it was important to determine the effect of metal ions other than cadmium on the reliability of the immunoassay. The potential interference by individual metals was examined over a wide range of concentrations. Standard curves were constructed by diluting cadmium at concentrations from 2 to 5000 ppb into solutions containing the indicated concentrations of the potentially interfering metal, as shown in Figures 3−6. A control standard curve was included which contained no metal other than cadmium. The metals examined were Pb(II), Mg(II), Fe(III), Ni(II) Zn-(II), Mn(II), In(III), and Hg(II). The data in the figures are based upon three determinations made at the same time. However, these curves have been reproduced at various times in the laboratory with essentially identical results. A summary of the effect of other metal ions on the cadmium immunoassay is presented in Table 1. The ability of a metal−EDTA complex to interfere in the competitive immunoassay

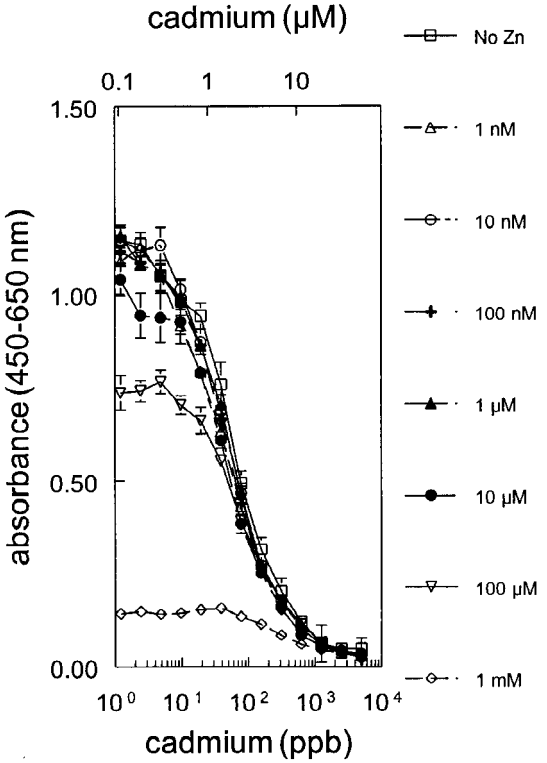

FIGURE 4. The effect of Zn(II) on the cadmium immunoassay. Competitive ELISAs were performed in the absence of added Zn (□), or in the presence of increasing Zn(II) concentrations as shown: (△) 1 nM; (○) 10 nM; (+), 100 nM; (▲), 1 μM; (●) 10 μM; (▽) 100 μM; (◇) 1 mM. Each point represents the mean of three determinations ± SD.

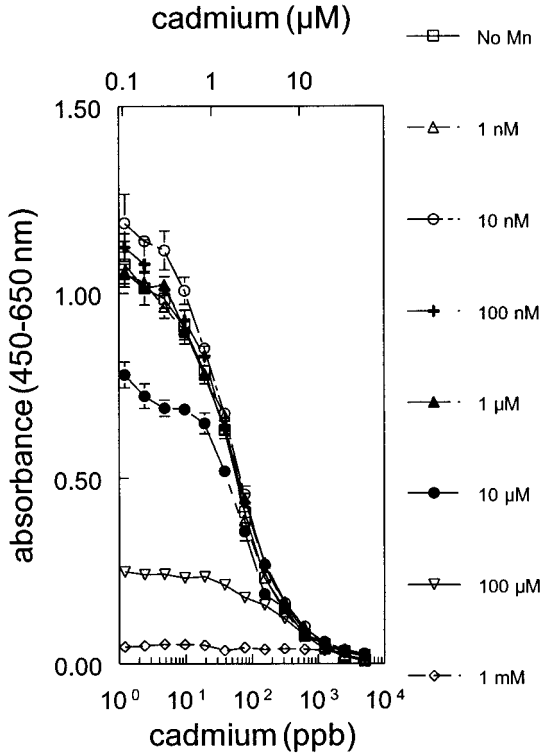

FIGURE 5. The effect of Mn(II) on the cadmium immunoassay. Competitive ELISAs were performed in the absence of added Mn (□), or in the presence of increasing Mn(II) concentrations as shown: (△) 1 nM; (○) 10 nM; (+) 100 nM; (▲), 1 μM; (●) 10 μM; (▽) 100 μM; (◇) 1 mM. Each point represents the mean of three determinations ± SD.

was well correlated with its affinity for the 2A81G5 monoclonal antibody (*12*).

The presence of Pb(II) at concentrations as high as 1 mM had no effect on the cadmium immunoassay, as shown in Figure 3. Experiments using Mg(II) and Fe(III) gave virtually identical sets of curves (data not shown), indicating that these cations will not interfere in the immunoassay. Previous studies have shown that the antibody binds to EDTA complexes of Au(III), Tb(III), Ga(III), and Al(III) less tightly than to the Pb(II)−EDTA complex (*12*); thus, it may be inferred that these metal ions will also have no effect on the cadmium immunoassay.

The immunoassay was relatively insensitive to Zn(II) interferences at concentrations up to 10 μM. At a concentration of 100 μM, Zn(II) began to inhibit color formation when the Cd(II) concentration in the immunoassay was below 1 μM. When the assay was run in the presence of 1 mM Zn(II), the color produced by the immunoassay was significantly depressed at all cadmium concentrations, as shown in Figure 4. The 2A81G5 monoclonal antibody binds to EDTA complexes of Zn(II) and Ni(II) with equivalent affinities (*12*) and the curves obtained using Ni(II) as the interfering cation were virtually identical to those generated with Zn(II) (data not shown). The immunoassay was also relatively insensitive to interferences by Mn(II) at concentrations up to 10 μM, as shown in Figure 5. Concentrations of 100 μM or higher caused significant interference in the assay. The monoclonal antibody binds to EDTA complexes of Mn(II) and In(III) with K_ds of 4.1×10^{-7} M and 6.2×10^{-7} M, respectively, and the interference curves obtained using In(III)−EDTA complexes were indistinguishable from those obtained using Mn(II) (data not shown).

The only metal which showed an ability to interfere with the immunoassay at concentrations likely to be present in field samples was Hg(II). As shown in Figure 6, the immunoassay was not affected by Hg(II) at concentrations of 1 μM; however, concentrations of 10 μM or higher caused significant positive interference in the immunoassay. The ability of the assay to give false positives in the presence of mercury concentrations above 1 μM indicates that those environmental samples which give a positive response in the assay should be reanalyzed by an independent method to distinguish between samples contaminated with ionic cadmium from those containing mercury.

In a final series of experiments, environmental water samples spiked with cadmium were provided as unknowns to laboratory personnel, who used the immunoassay to analyze them for cadmium content. The samples were independently analyzed for cadmium content using a graphite furnace atomic absorption spectrophotometer (GFAAS); the limit of detection of cadmium by GFAAS in bayou water was 0.5 ppb. A comparison of the results obtained from the two methods are shown in Figure 7. The results from the immunoassay correlated well with the values obtained from GFAAS and the immunoassay correctly identified minimally, moderately, and heavily contaminated water samples. There was some positive bias in the immunoassay as indicated by the nonzero intercept of the graph in Figure 7; however, such a positive bias may be acceptable in an assay designed to be used as a field portable screening test.

The cadmium immunoassay described in this communication had the ability to detect Cd(II) in ambient water samples at levels from approximately 10 to 2000 ppb in 1−2 h. The equipment required for the analysis was limited to test tubes, microtiter plates, pipettors, and an ELISA plate reader. Although the data presented herein utilized a laboratory-based plate reader, similar cadmium standard curves have been generated with a battery-operated plate

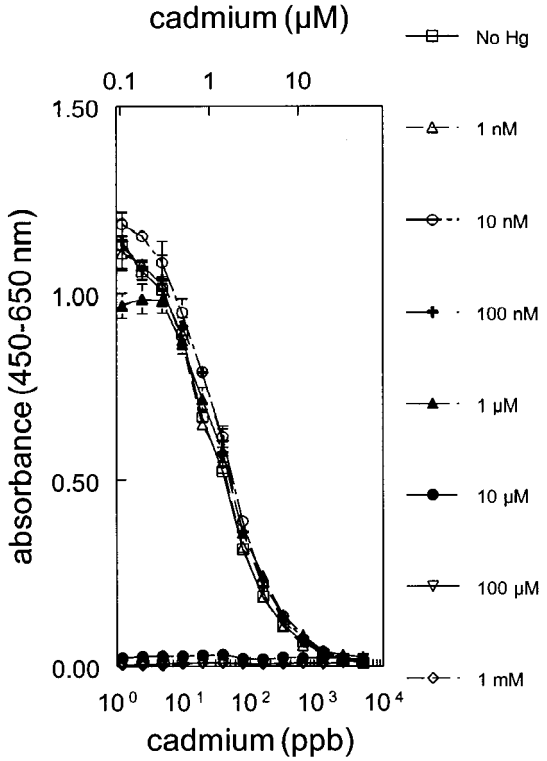

FIGURE 6. The effect of Hg(II) on the cadmium immunoassay. Competitive ELISAs were performed in the absence of added Hg (□), or in the presence of increasing Hg(II) concentrations as shown: (△) 1 nM; (○) 10 nM; (+) 100 nM; (▲) 1 μM; (●) 10 μM; (▽) 100 μM; (◇) 1 mM. Each point represents the mean of three determinations ± SD.

TABLE 1. Affinity of a Metal−EDTA Complex for the 2A81G5 Antibody Correlates with Its Inhibitory Effect in Competitive Immunoassay

metal−EDTA complex	equilibrium dissociation constant (M)[a]	inhibitory concentration in immunoassay (M)[b]
Mg(II)	2.2×10^{-4}	$>1 \times 10^{-3}$
Pb(II)	7.4×10^{-5}	$>1 \times 10^{-3}$
Fe(III)	5.4×10^{-5}	$>1 \times 10^{-3}$
Zn(II)	2.5×10^{-6}	1×10^{-3}
Ni(II)	2.1×10^{-6}	1×10^{-3}
Mn(II)	4.1×10^{-7}	1×10^{-4}
In(II)	6.2×10^{-7}	1×10^{-4}
Hg(II)	2.6×10^{-8}	1×10^{-5}

[a] Binding affinities of antibody for metal−EDTA complexes are from ref 12. [b] Concentration of metal−EDTA complex which inhibited color formation in the competitive immunoassay by >50%.

reader suitable for use at the site of contamination (data not shown). The limit of detection, defined as two standard deviations above the minimum detectable limit, is approximately 7 ppb cadmium. Because the antibody used in this assay recognizes the Cd(II)−EDTA complex but not metal-free EDTA, the assay may be run in a large molar excess of EDTA. This large excess of EDTA has two advantages in the assay: (1) it ensures that all metal cations present in the environmental sample will be complexed with EDTA, and (2) it competes metal cations from any organic metal-binding molecules that may be present in the environmental samples. Experiments are in progress to reformat this immunoassay for the analysis of soil samples.

The availability of field portable tests for the detection of heavy metals in environmental samples is very limited. BioNebraska manufactures a test kit for the determination of inorganic mercury in soils (BiMelyze) which utilizes an antibody with a primary specificity for Hg(II) coupled to glutathione (*10*). The assay reported a sensitivity of 2−2.5 ppm in soil samples. Portable X-ray fluorescent instruments have also been used on-site to detect metals directly in the soil (*19*). Although these instruments are not very sensitive (detection range, 20−100 ppm, depending upon the metal), they do have the ability to detect the presence of several metals without disturbing the soil surface.

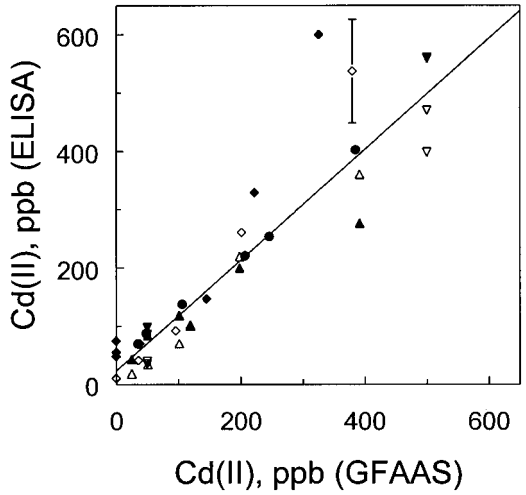

FIGURE 7. Comparison of ELISA and atomic absorption results in the analysis of cadmium in environmental water samples. ▽, △, and ◇ samples prepared in reagent water; ●, ▼, ▲, and ◆ samples prepared in water from Bayou Trepagnier. Linear regression analysis generated a line with a slope of 0.951, an intercept of 23.07 ± 9.5, and a correlation coefficient of 0.931.

Heavy metals are of particular concern to both the general public and regulatory agencies because of their persistence in the environment and their ability to be mobilized by changes weather patterns and hydrology. Immunoassay techniques are attractive to local, state, and federal agencies because they permit rapid sample screening and lower the total cost of analysis. Immunological screening methods have now been approved by regulatory agencies for a variety of analytes (*20*, *21*). The availability of immunoassays for heavy metals will permit the analyst to rapidly screen for contamination and determine if plumes of mobilized metals exist in specific areas. The immunoassay described herein is the first in a series from this laboratory designed to provide broad screening for metals which may pose a threat to the environment.

Acknowledgments

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