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Development and Validation of a One-Step Immunoassay for Determination of Cadmium in Human Serum

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A sensitive and simple one-step immunoassay was developed and validated for quantitative determination of Cd(II) in human serum. In this method, a monoclonal antibody that recognizes Cd(II)−EDTA complexes was directly immobilized onto microwell plates. The serum sample containing metallothionein(MT)-bound and non-MT-bound Cd(II) was acidified to displace the Cd(II) from MT. The sample was then treated with metal-free EDTA to convert Cd(II) to Cd(II)−EDTA complexes. A mixture of Cd(II)−EDTA complexes derived from serum samples and Cd(II)−EDTA conjugated with peroxidase enzyme was incubated in the wells to compete for binding sites of the immobilized antibody. After addition of peroxidase substrate, the bound fraction of the enzyme conjugate was measured by a microplate reader, and the signal was inversely proportional to the concentration of the Cd(II) in the sample. The assay limit of detection was 0.24 μg/L, and the effective working range at coefficient of variation of ≤10% was 0.24−100 μg/L. Analytical recovery of spiked Cd(II), in the concentration range between 0.8 and 50 μg/L, was 97.8 ± 4.0%. The assay was selective for Cd(II); other metal ions (Mn, Co, Cu, Zn, Mg, Hg, Ca, Ni, Fe, and Pb), tested at concentrations considerably higher than those present in human serum, did not significantly interfere with the assay. The assay results correlated well with those obtained by graphite furnace atomic absorption spectrometry ($r = 0.984$).

Cadmium is an environmental and occupational hazard that can cause serious health problems.[1] In humans, the half-life time of cadmium exceeds 10 years, and the organs most sensitive to cadmium exposure are the kidneys. The first sign of toxicity is tubular damage with an increase of urinary excretion of small proteins.[2−4] Excessive exposure causes more progressively severe and irreversible renal damage and secondary effects on the mineral and calcium metabolism.[5,6] In addition, experimental and epidemiological studies are providing substantial evidence that low levels of long-term exposure to cadmium can contribute to an increased risk of cancer.[7,8] The toxicity of cadmium is related to its chemical form, physical state, and oxidation state; the inorganic species of cadmium are more toxic than the organic ones, and among the inorganic ones, the bivalent oxidation state is the most toxic.[1] Dose−response relationships between human exposure to cadmium and renal dysfunction have been well-established,[9,10] and legislation has been enacted to reduce human exposure to cadmium.[11−13] In the United States, the acute and chronic exposure criteria for dissolved cadmium in freshwater are 3.7 and 1.0 μg/L, respectively; in marine waters, the acute and chronic cadmium exposure criteria are 9.3 and 4 μg/L, respectively.

A number of investigations have detected early stages of cadmium-induced renal dysfunction by examining different urinary indicators;[14−16] however, blood cadmium level is a more relevant and specific dose estimate for exposure.[17,18] The availability of an

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analytical technique that could provide near-real-time data on cadmium levels in blood samples could be a useful tool for assessment of recent human exposure to cadmium and could ultimately reduce the incidence of human intoxication. In non-occupationally exposed subjects, the cadmium concentration in blood is generally <5 μg/L.[19,20] Of the whole blood cadmium concentration, only 10% is circulating in the serum.[21] Serum is a more convenient, homogeneous sample to collect and store, and it is not subject to the degradation that can occur in whole blood. The availability of a simple, sensitive test for cadmium in serum could permit the analysis of stored serum banks and could allow epidemiological studies of cadmium exposure in populations. Recent studies of serum cadmium levels in pediatric age groups have linked elevated serum Cd to environmental tobacco smoke exposure.[22,23] Cadmium in serum exists primarily as the metallothionein-bound form, and studies have demonstrated that Cd−metallothionein complexes are toxic to the kidney and central nervous system.[24,25] The availability of an immunoassay that selectively measures serum/metallothionein-bound cadmium could lead to new insights into the mechanisms of toxicity of this heavy metal.

Electrothermal atomic absorption spectrometry (ET-AAS) is the most commonly used analytical method for quantitative determination of metals. The routine limit of detection for cadmium in biological fluids is ~2 μg/L, although the addition of appropriate modifiers and Zeeman correction can decrease the limit of detection by approximately 10-fold.[26−31] Even lower levels of detection have been achieved using inductively coupled plasma-mass spectrometry.[32−34] These techniques accurately measure the concentrations of cadmium; however, they require expensive instruments in a centralized facility, and sample turnaround is relatively slow. Research is, therefore, needed for development of new technology with rapid turnaround times, improved simplicity, and lower cost for measurement of serum and blood cadmium levels.

In our previous work, we demonstrated immunoassays as a reliable alternative approach for analysis of cadmium ions in environmental water samples.[35,36] These assays are remarkably quick, easily performed, require minimum sample pretreatment, have short turnaround time, and are cost-effective. In general, such immunoassays employed either plate-bound antigen [36] or plate-bound antibody[35] formats. In the later format, the immobilized antibody provided the binding sites for the enzyme-labeled metal ion and competing metal ions in the sample. This assay was carried out in one binding step, and the enzyme-labeled metal ions were used for generation of the signal. The present study is focused on development and validation of a one-step plate-bound antibody immunoassay for the determination of Cd(II) in human serum at concentrations as low as 0.24 μg/L. This assay used a previously described monoclonal antibody that recognizes Cd(II)−EDTA complexes but not metal-free EDTA[37] and employed a peroxidase conjugate of Cd(II)−EDTA as an enzyme label.[35]

EXPERIMENTAL SECTION

Materials. Metallothionein II (MT: from rabbit liver, contains 1.5% zinc and 7.8% cadmium), horseradish peroxidase (HRP) enzyme (EC 1.11.1.7, type X), protease-free bovine serum albumin (BSA), ethylenediamine *N,N,N',N'*-tetraacetic acid (EDTA), and Sephadex G-50 were purchased from Sigma Chemical Co. (St. Louis, MO). 1-(4-Isothiocyanobenzyl)-ethylenediamine *N,N,N',N'*-tetraacetic acid (ITCBE) was purchased from Dojindo Laboratories (Gaithersburg, MD). Cadmium foil (99.999%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Atomic absorption spectroscopy standard metals (1000 mg/L in 2% HNO_3) were obtained from Perkin-Elmer Corporation (Norwalk, CT). A pooled human serum sample was purchased from Intergen Co. (Milford, MA). This serum was shown to be Cd(II)-free by atomic absorption spectrometry before use. Ultrapure metal-free hydrochloric and nitric acids were purchased from Fisher Scientific Co. (Houston, TX). 3,3′,5,5′-Tetramethylbenzidine peroxidase substrate (TMB Microwell substrate) was from Kirkegaard-Perry Laboratories (Gaithersburg, MD). ELISA high-binding microwell plates, and "sealing tape", i.e., adhesive-backed plastic film to cover and seal off the plates, were obtained from Corning/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 and liberally rinsed with purified water, and all plasticware was soaked overnight in 3 M HCl and rinsed liberally with purified water before use. Amicon Centricon YM-3 concentrator tubes (Millipore Co., Bedford, MA) were treated with 100 mM EDTA and liberally rinsed with water before use.

Preparation of the Antibody and Enzyme-Labeled Conjugate. The monoclonal antibody (2A81G5) was generated by fusing SP2/0-Ag14 mouse myeloma cells with spleen cells from a

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BALB/c mouse immunized with Cd(II)−EDTA conjugated to keyhole limpet hemocyanin. Our laboratory has previously described the isolation, purification, and characterization of this antibody.[37] The cadmium horseradish peroxidase enzyme conjugate (Cd(II)−EDTA−HRP) was prepared by reacting the isothiocyanato group of Cd(II)-loaded ITCBE with the lysine ϵ-amino groups of the peroxidase enzyme. We previously described the preparation and characterization of this conjugate.[35]

Gel Filtration of EDTA-Treated MT. Reaction mixtures containing 200−1000 μg/mL MT and 5 mM EDTA were allowed to react for 1 h at 25 °C. After incubation, 0.5 mL aliquots were applied to a Sephadex G-50 column (1.5 × 30 cm) equilibrated at room temperature with HEPES-buffered saline (HBS: 137 mM NaCl, 3 mM KCl, and 10 mM HEPES, pH 7.4). Fractions (0.5 mL) were collected and monitored spectrophotometrically at 254 nm for identification of the chromatographic peaks corresponding to the MT protein and EDTA. The distribution of Cd(II) between MT and EDTA was examined by the testing the reactivity of the column fractions by immunoassay.[35]

Ultrafiltration of Acid-Treated MT. A series of reaction mixtures containing MT in the concentration range of 0.01−1000 μg/L Cd(II) were prepared in HBS. The samples were mixed (1:1) with 1 M HCl and allowed to react for 5 min at 25 °C. The reaction mixtures were subjected to ultrafiltration using a 3 kDa cut-off filter (Centricon YM-3). During centrifugation (10 min, 1900*g*, 4 °C) the filters completely retained the apo-MT, but the displaced Cd(II) passed through the filter membrane. The filtrate solutions were subsequently neutralized with KOH and their Cd(II) concentrations were determined by immunoassay.[35]

Coating of Microwell Plates. Purified 2A81G5 antibody was diluted in HBS at a concentration of 2.5 μg/mL, and 50 μL of the diluted solution was introduced into each well. The plates were covered with a sealing tape and incubated at 37 °C for 2 h. The plates were washed three times with phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, and 10 mM sodium phosphate, pH 7.4) containing 0.05% Tween 20. The wells were blocked with 3% bovine serum albumin (BSA) in HBS by incubation at 37 °C for 1 h, followed by a washing step. The plates could be used on the same day or covered with sealing tape and stored for at least 4 weeks at 4 °C. After storage, the plates were washed once with PBS containing 0.05% Tween 20 before use.

Immunoassay Procedure. All procedures were carried out at 25 °C. A 75-μL aliquot of serum was mixed (1:1) with 5% HNO_3, vortexed, and incubated for 5 min at room temperature. The samples were centrifuged for 5 min at 15 000 rpm. The supernatants were conditioned by the addition of a 10% volume of a concentrated buffer solution containing 1.37 M NaCl, 30 mM KCl, 50 mM EDTA, and 100 mM HEPES, pH 7.4. The solutions were neutralized with 10 M KOH and subsequently mixed (1:1) with 0.1 μg/mL Cd(II)−EDTA−HRP conjugate solution containing 1% BSA. Aliquots (50 μL) of the mixture were added to a microwell that had been previously coated with 2.5 μg/mL of 2A81G5 antibody and blocked with 3% BSA. After a 1-h incubation, the plates were washed three times with PBS containing 0.05% Tween 20. TMB microwell substrate (50 μL/well) was used for color development. After stopping the color developing reaction with 1 N HCl (50 μL/well), the absorbance of each well was measured in a dual wavelength mode (450−650 nm) using a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). The data were transformed to a four-parameter curve using SoftMax software provided with the instrument.

Data Analysis. Values for IC_{50} were those that gave the best fit to the following equation

$$A = A_0 - \{(A_0 - A_1)[\mathrm{Cd(II)}]/(\mathrm{IC}_{50} + [\mathrm{Cd(II)}])\}$$

where A is the signal at a definite known concentration of Cd(II), A_0 is the signal in the absence of Cd(II), A_1 is the signal at the saturating concentration of Cd(II), and IC_{50} is the Cd(II) concentration that produces a 50% inhibition of the signal. The concentrations of Cd(II) in the samples were then obtained by interpolation on the standard curve. The standard curve for Cd(II) was generated using atomic absorption grade Cd(II) by the same procedure on plates of the same series.

RESULTS AND DISCUSSION

This study describes a sensitive one-step enzyme immunoassay that quantifies Cd(II) in human serum samples. Figure 1 illustrates the general principles of this assay. The serum sample, containing MT-bound, non-MT-bound Cd(II), or both was acidified for displacement of MT-bound Cd(II). The displaced and non-MT-bound Cd(II) were captured by a molar excess of metal-free EDTA to ensure that all of the Cd(II) in the sample was present as an EDTA complex, the form recognized by the antibody. This solution was neutralized, mixed with Cd(II)−EDTA−HRP conjugate, and subsequently incubated with the immobilized antibody in the microwell. During this incubation, the Cd(II)−EDTA complexes competed with the Cd(II)−EDTA−HRP conjugate for binding sites of the immobilized antibody. After removal of unbound reagents, the amount of enzyme conjugate bound to the antibody was determined using a chromogenic substrate. The concentration of Cd(II) in a sample was quantified by the ability of its EDTA complex to inhibit the binding of Cd(II)−EDTA−HRP conjugate to the antibody, and the color development was inversely proportional to the concentration of Cd(II) in the original sample.

Optimum Assay Conditions. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES) was chosen as the buffer in the present work because of its negligible metal-binding capacity.[38] The optimum concentration of 2A81G5 antibody required for coating onto the microwell plates and the best working concentration of the Cd(II)−EDTA−HRP enzyme conjugate that gave the most sensitive assay were determined in our previous work.[35] These concentrations were 2.5 and 0.1 μg/mL, respectively, for the 2A81G5 antibody and the Cd(II)−EDTA−HRP conjugate. For more convenience in clinical testing, it was important to check the stability of the 2A81G5 antibody after its coating onto the microwell plates. Plates were coated with the antibody and stored for varying periods of time at 4 °C and then were analyzed for the amount of active antibody remaining on the microwells. As shown in Figure 2, the plates could be stored for at least 4 weeks. This gives an advantage that the plates could be kept, after coating with the antibody and blocking with BSA, until the assay time. This reduces 3 h from the total time required for analysis. If the

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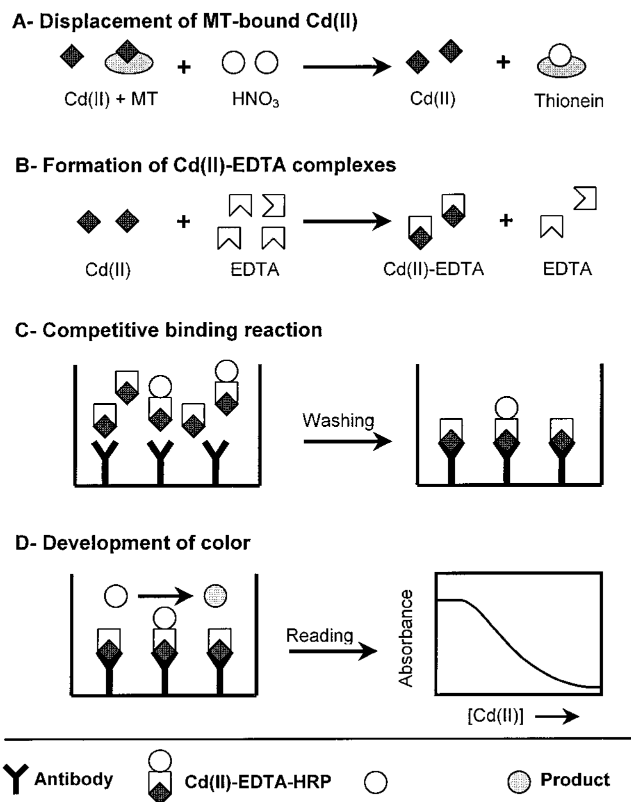

Figure 1. Schematic diagram of the competitive immunoassay for Cd(II) in serum. (A) Serum sample containing MT-bound and MT-non bound Cd(II) is treated with 5% HNO_3 to displace the MT-bound Cd(II). (B) The Cd(II) ions are captured by a molar excess of metal-free EDTA. (C) The solution containing the Cd(II)−EDTA complexes is mixed with the Cd(II)−EDTA−HRP enzyme conjugate, and the mixture is incubated with the immobilized 2A81G5 antibody in the microwell. During this incubation, the Cd(II)−EDTA complexes compete with the enzyme conjugate for binding sites of the immobilized antibody. Any unbound reagents are removed by a wash step. (D) After removal of unbound reagents, the amount of enzyme conjugate bound to the antibody is determined using a chromogenic substrate. The concentration of Cd(II) in a sample is quantified by the ability of its EDTA complex to inhibit the binding of Cd(II)−EDTA−HRP conjugate to the antibody, and color development is inversely proportional to the concentration of Cd(II) in the original sample.

plates are to be stored for extended periods of time (>2 weeks), it is important to use a high purity BSA (substantially protease- and metal-free) during the blocking procedure.

Reaction of Metallothionein with EDTA. Metallothionein (MT) is a low-molecular-weight protein that is induced by exposure to metals;[39−41] it has a high affinity to Cd(II).[42] Cd(II) accumulates in the body mainly as the MT-bound form. Non-MT-bound Cd(II) can be detected before induction of sufficient MT to sequester Cd(II) or after accumulation of Cd(II) in amounts beyond the capacity of MT for Cd(II) binding.[19] The non-MT-bound Cd(II) can be readily analyzed by immunoassay in samples

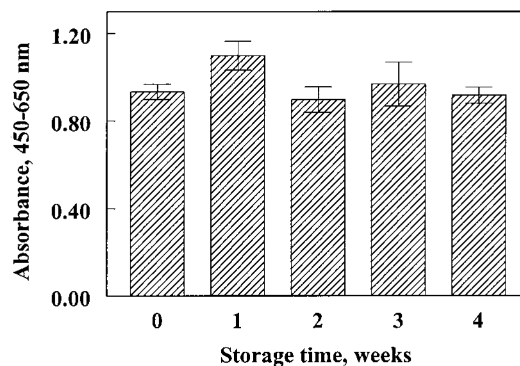

Figure 2. The stability of 2A81G5 antibody coated onto the microwell plates. The purified antibody was coated onto the microwells at 2.5 μg of protein/mL, and the wells were subsequently blocked with 3% BSA. The plates were stored at 4 °C for varying periods of time and then analyzed for the amount of active antibody remaining on the microwells. Values are the mean of eight determinations ± SD.

treated with EDTA to form Cd(II)−EDTA complexes, the form recognized by the antibody; however, the MT-bound Cd(II) should be completely removed from MT before analysis in the immunoassay. Removal of Cd(II) from MT in ligand substitution processes has been previously studied with EDTA as a competing ligand.[43−45] These studies indicated that reaction with EDTA could remove Cd(II) from MT; however, the reaction is dependent on the EDTA concentration, and it occurs with slow kinetics.[46] Therefore, it was necessary to investigate whether Cd(II) could be completely removed from MT under the assay conditions employed herein: 1-hr incubation with 5 mM EDTA. A reaction mixture containing 1000 μg/mL of MT was allowed to react for 1 h with 5 mM EDTA, and then an aliquot was applied to a Sephadex G-50 column to examine the distribution of Cd(II) between the MT- and EDTA-bound forms. As shown in Figure 3, there were two immunoreactive Cd(II)-containing peaks in the column eluate, one representing MT-bound Cd(II) and the other corresponding to Cd(II) released from MT and bound to EDTA. Similar results were obtained when 200 μg/mL MT was treated under identical conditions (data not shown). These results indicated that Cd(II) was not completely removed from MT under the conditions employed in the immunoassay.

Displacement of MT-Bound Cd(II) by Acid. In previous studies, acidification was used for removal of Cd(II) from MT.[47] We applied this approach in the present study. A series of reaction mixtures containing MT in the concentration range of 0.01−1000 μg/L Cd(II) were prepared and acidified with 1 M HCl. Ultrafiltration using nominal 3 kDa molecular weight cut-off filters was used to separate the displaced Cd(II) from the MT protein. Concentrations of the displaced Cd(II) in each filtrate were determined by immunoassay[35] using atomic absorption grade Cd(II) as a reference standard. As shown in Figure 4, the values

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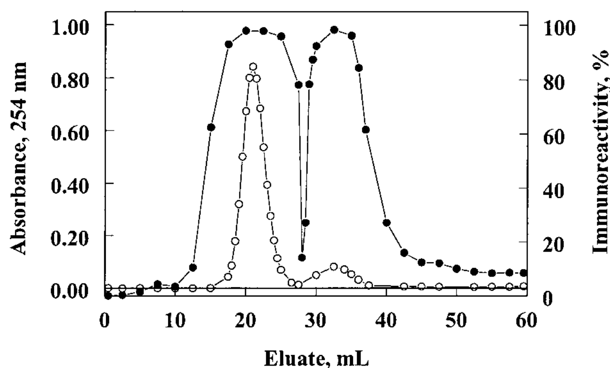

Figure 3. Size exclusion chromatography of EDTA-treated MT. A reaction mixture containing 1.0 mg/mL MT and 5 mM EDTA was allowed to incubate for 1 h at 25 °C. After incubation, a 0.5-mL aliquot was applied to a Sephadex G-50 column (1.5 × 30 cm) equilibrated with HBS at pH 7.4 at room temperature and fractions were collected. The absorbance at 254 nm (○) and immunoreactivity for Cd(II) (●) were monitored for each fraction.

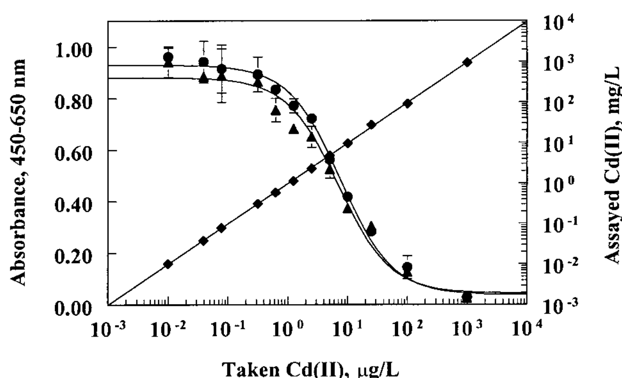

Figure 4. Displacement of MT-bound Cd(II) by low pH. Reaction mixtures containing MT-bound Cd(II) and 1 M HCl were allowed to react for 5 min at 25 °C. After incubation, ultrafiltration (nominal 3 kDa molecular weight cut-off filter) was used to separate the released Cd(II) from the Cd(II)−MT. The filtrates were neutralized, and their Cd(II) concentrations were determined by immunoassay. The data obtained using MT-bound Cd(II) (▲) were compared with those obtained by subjecting equivalent concentrations of atomic absorption grade Cd(II) to identical acidification and ultrafiltration procedure (●). A comparison of Cd(II) concentrations found in each filtrate solution, in micrograms per liter, was shown (♦). Values are mean ± SD of duplicate determinations.

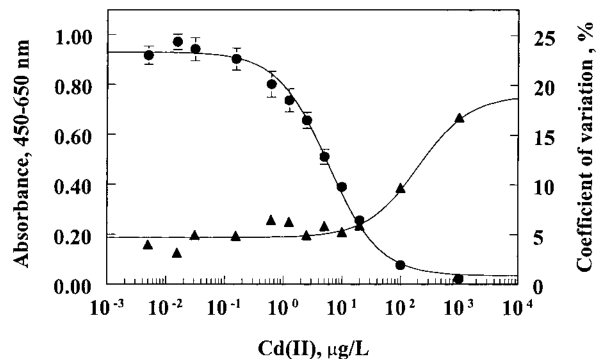

Figure 5. Calibration curve (●) and precision profile (▲) of the Cd(II) immunoassay in serum. Microwells were coated with 2.5 μg/mL of purified 2A81G5 antibody. Varying concentrations of atomic absorption grade Cd(II) were prepared in Cd(II)-free serum and pretreated as described in the Experimental Section. The samples were mixed (1:1) with Cd(II)−EDTA−HRP conjugate, and then the mixture was introduced into each microwell. The microwells were further manipulated as described in the Experimental Section. The values plotted are mean ± SD of eight determinations.

obtained for the Cd(II) displaced from MT are comparable to those obtained using atomic absorption grade Cd(II). In addition, the amounts of displaced Cd(II) found in the filtrate solutions were excellently correlated ($r = 0.999$) with the amounts of MT-bound Cd(II) taken for the analysis. These results confirm the complete removal of MT-bound Cd(II) by acidification with 1 M HCl. Similar results were obtained when 5% HNO_3 was used for acidification (data not shown). All subsequent serum-involving experiments were performed using 5% HNO_3 for acidification because HNO_3 was more effective than HCl in precipitating extraneous protein from the reaction mixture.

Analytical Performance of the Assay. The calibration curve for Cd(II) immunoassay in serum is shown in Figure 5. This curve was generated by adding atomic absorption grade Cd(II) at concentrations from 0.0 to 1000 μg/L to Cd(II)-free serum and treating the samples as described in the Experimental Section. The assay sensitivity, defined as the slope in the middle of the calibration curve, was −1.38 ± 0.55. The assay limit of detection was determined by identifying the lowest measurable concentration of Cd(II) that could be distinguishable from zero concentration ± 3 SD. On the basis of eight replicate measurements, the limit of detection was 0.24 μg/L Cd(II) in serum.

The assay precision profile, obtained from the results of calibration standard samples, is shown in Figure 5. The assay gave satisfactory results; the coefficients of variations over the entire linear range of the assay were <10%. In general, the precision in competitive immunoassays depends mainly upon the uniformity in the quantity of the coated reagent from well to well in a microwell plate. Any interference in this uniformity could arise from the experimental protocol and other manipulations, change in the temperature of incubation, and dispensing the reagents. In the present assay, the short period of incubation and minimum manipulations at 25 °C contributed to good precision.

Recovery of the assay was assessed by adding varying known amounts (0.8−50 μg/L) of MT-bound Cd(II) to Cd(II)-free serum samples; each sample was subsequently analyzed in triplicate for its Cd(II) content. The mean analytical recovery was calculated as the ratio between the Cd(II) concentration found and the concentration added, expressed as percentage. As shown in Table 1, a quantitative recovery (94.1 ± 2.4% to 105.7 ± 6.9%) of the added Cd(II) was obtained, indicating the accuracy of the method and an absence of endogenous interfering substances in serum samples.

The dilution linearity of the assay response was tested by analyzing a serum sample spiked with 100 μg/L MT-bound Cd(II) and diluted with Cd(II)-free serum. An excellent linear relationship ($y = 1.027 + 0.862x$; $r = 0.997$) was found between the theoretical amounts of Cd(II) and the amounts found by assay (Figure 6).

The selectivity of the assay for Cd(II) was studied in the presence of other metal ions (Mn, Co, Cu, Zn, Mg, Hg, Ca, Ni, Fe, and Pb) at concentrations considerably higher than those present in human serum. As shown in Figure 7, the cross reactivity exhibited by any of the tested metals was <2%. Although it has

Table 1. Analytical Recovery of Cd(II) Added to Serum Sample

added Cd(II), μg/L	found Cd(II), μg/L	recovery (%)
0.80	0.76 ± 0.20[a]	97.5 ± 2.4
1.56	1.53 ± 0.02	97.9 ± 1.5
3.13	3.04 ± 0.13	97.2 ± 4.3
6.25	5.88 ± 0.15	94.1 ± 2.4
12.50	12.03 ± 0.22	96.3 ± 1.7
25.00	26.42 ± 1.74	105.7 ± 7.0
50.00	48.04 ± 4.24	96.1 ± 8.5
ave		97.8 ± 3.9

[a] Values are mean of three determinations ± SD.

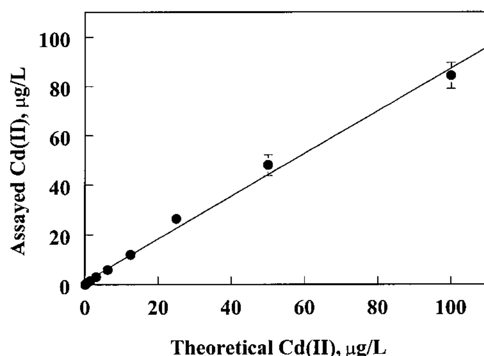

Figure 6. Dilution test of the Cd(II) immunoassay in serum. A serum sample spiked with MT-bound Cd(II) to a final concentration of 100 μg/mL Cd(II) was diluted with Cd(II)-free serum and then analyzed by immunoassay as described in the Experimental Section. The assayed Cd(II) concentrations were compared with the theoretical concentrations. Values are mean ± SD of triplicate determinations.

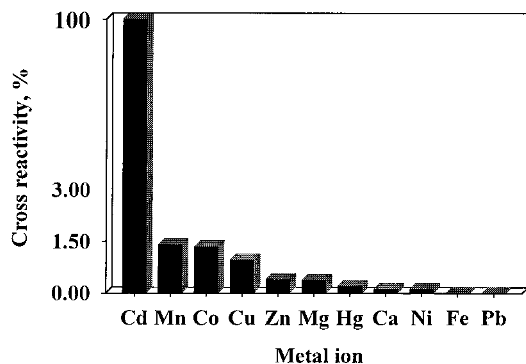

Figure 7. Metal ion specificity of the Cd(II) immunoassay in serum. Competitive immunoassays were performed in duplicate, as described in the Experimental Section, using atomic absorption grade metal ions. Cross-reactivity was calculated as the IC_{50} [metal ion]/IC_{50}[Cd(II)] × 100, where the IC_{50} is the concentration of metal−EDTA complex that inhibits the color formation in the competitive immunoassay by 50%.

been shown that 2A81G5 antibody bound to Cd(II)− and Hg(II)−EDTA complexes with approximate equal affinity (equilibrium dissociation constants were 21 and 26 nM, respectively),[37] its cross-reactivity in the present assay was negligible. This is due to the high BSA concentration employed in the assay, which strongly binds to Hg(II)[48] and prevents its binding the antibody in the assay.[35]

(48) Alexander, M.; Berthold, H. *Anal. Lett.* **1998**, *31*, 1633−50.

Table 2. Comparison of Immunoassay with Atomic Absorption Spectroscopy for Analysis of Serum Samples Spiked with Cd(II)[a]

	found Cd(II), μg/L	
spiked Cd(II), μg/L	immunoassay	atomic absorption
0.31	0.28 ± 0.01[b]	0.19 ± 0.01
0.63	0.55 ± 0.04	0.40 ± 0.01
1.25	1.64 ± 0.08	0.76 ± 0.05
2.5	2.09 ± 0.28	1.90 ± 0.15
5.0	6.42 ± 0.78	4.88 ± 0.03
10	13.89 ± 1.20	9.98 ± 0.80
20	18.09 ± 1.17	21.96 ± 0.32
40	44.92 ± 2.97	38.64 ± 2.63

[a] Samples were spiked with metallothionein-bound Cd(II) and treated as described in the Experimental Section for analysis by immunoassay. [b] Values are mean of triplicate determinations ± SD.

Application and Comparison of the Assay. A series of Cd(II)-spiked serum samples were prepared in the concentration range from 0.31 to 40 μg/L by diluting a MT-bound Cd(II) sample into a Cd(II)-free pooled human serum sample. These samples were analyzed for Cd(II) both by the immunoassay procedure described in the Experimental Section and by graphite furnace atomic absorption spectrometry (GF-AAS). The comparison of these results is shown in Table 2. The absolute limit of detection for cadmium on this instrument with standard samples was 0.2 μg/L. Although the AAS method was not able to accurately determine spikes lower than 2 μg/L in the serum matrix, the overall method comparison was satisfactory in terms of the correlation coefficient, calculated for the two entire data sets, indicating the ability of the immunoassay to accurately determine Cd(II) at each concentration tested. Linear regression analysis of the results yielded a linear equation: $y = 0.312 + 1.086x$, $r = 0.984$.

CONCLUSION

A sensitive one-step immunoassay for determination of Cd(II) in serum samples has been successfully developed and validated. The assay was made possible by using an immobilized monoclonal antibody with high binding affinity for Cd(II)−EDTA complexes as the solid phase, and Cd(II)−EDTA−HRP conjugate as an enzyme label. The pretreatment of the serum sample for the assay was minimal. The assay employed only one incubation step, after which the plate was ready to generate the signal. The assay is very easy to perform in a 96-well plate, and it is not time-consuming; an operator can analyze a batch of 20 samples in triplicate and obtain the results of analysis in less than 2 h. In addition, the assay described in this study exhibits an excellent limit of detection: it is able to detect Cd(II) in serum samples at concentrations as low as 0.24 μg/L. Because almost all current biomonitoring data for cadmium is presented as total elemental analysis, our first priority was to demonstrate that the immunoassay provides data equivalent to that collected by AAS. Future studies will include the exploitation of this approach for cadmium speciation. Our preliminary experiments have shown that the method may be applicable to whole blood analysis, as well. Immunoassays generally have faster turnaround times than other analyses, because the multiwell format permits simultaneous

analysis of multiple samples. ET-AAS and other instrument-intensive analyses require sequential analysis, and samples often sit in a queue for long periods of time. When time-in-queue is taken into account, immunoassays are almost always faster than analyses requiring expensive instrumentation. The assay is an answer to the need for improved analytical techniques for measuring Cd(II), as the World Health Organization strongly recommends. To our knowledge, this assay represents the first immunoassay for the analysis of heavy metals in biological fluids.

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