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# Development of MAb-based Immunochromatographic Assay for Cadmium from Biological Samples

Kazuhiro Sasaki, Kei Tawarada, Hirosi Okuhata, Hitoshi Miyasaka, Akira Okuyama, Yukinao Maruyama, Naoya Ohmura

***Abstract*—Cadmium (Cd) is a general environmental pollutant of increasing global concern. In 2005 the joint FAO/WHO Codex Alimentarius Commission proposed new international food legislation for low-level Cd contaminants. In this study we demonstrate the use of novel monoclonal antibody (MAb) to Cd-EDTA in an immunochromatography (IC) format for the quick testing for trace Cd. This IC device could detect 0.3 μg $kg^{-1}$ (0.3 ppb) Cd. Contaminated Zn, Mn, Mg, and Cu, which would interfere the measurement of Cd by cross reaction to the MAb, could be removed by using a column that could separate trace Cd from other heavy metals in the extract of brown rice**

## I. Introduction

In the last ten years, several laboratories have begun to study bio-sensing for heavy metals. The studies of monoclonal antibodies (MAbs) toward heavy metals have been successful in targeting metal-chelate complex, for example Cd-EDTA [1] and Pb-DTPA [2]. With the heavy metal, bifunctional chelators, e.g. Isothiocyanobenzyl-EDTA, covalently conjugated to a carrier protein was shown to elicit an immune response [3]. Mabs to Cd-EDTA was applied to assays for detecting Cd in environmental water and human serum [4], [5]).

## II. Materials and Methods

### A. Materials

BALB/cA Jcl inbred mice were purchased from Clea Japan, Inc. (Tokyo, Japan). 1-(4-Isothiocyanobenzyl) ethylenediamine -N,N,N',N'-tetraacetic acid (Isothiocyanobenzyl-EDTA) was obtained from Dojindo (Kumamoto, Japan). Keyhole limpet hemocyanin (KLH) and Ovalbumin (OVA) were obtained from Sigma-Aldrich (A2512 and H7017; St. Louis, MO). Cy-5 conjugated F(ab')2 fragment of goat anti mouse IgG #286402 was obtained from Jackson ImmunoResearch (West Grove, PA). Myeloma cell (NS0) was purchased from The Institute of Physical and Chemical Research Cell Bank (Tsukuba, Japan).

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K. Sasaki and N. Ohmura are with Central Research Institute of Electric Power Industry, 270-1194 Japan (phone: +81-471-82-1181; fax: +81-471-83-4437; e-mail: k-sasaki@ criepi.denken.or.jp).

K. Tawarada, H. Okuhata and H. Miyasaka are with the The Kansai Electric Power Co., Inc., Seika-cho, Japan

A. Okuyama and Y. Maruyama are with EnBioTec Laboratories Co., Ltd, Tsukuba, Japan .

### B. Preparation of Protein-Chelate Conjugates

Preparation of protein-chelate complexes was based on previous report [1]. Briefly, 2 mg protein (KLH or OVA) was dissolved in 3 ml of 100 mM boric acid (pH 9.0) and then mixed with 1 mg isothiocyanobenzyl-EDTA over night. During this time, isothiocyanobenzyl-EDTA bound with the amino group of the protein. Before coordinating lead to conjugated EDTA, the pH of the solution was adjusted to 7.0 using a desalting column (10DG; Bio-Rad Laboratories; Hercules, CA) to prevent metal precipitation. To make Pb-EDTA-protein conjugate, 80 μl of 10 μM PbCl2 solution was added to 4 ml of 1mg / ml EDTA-protein solution.

### C. Immunization of Mice and Hybridoma Production

Three mice were injected intraperitoneally at 2 week intervals with Pb-EDTA KLH conjugate emulsified in adjuvant (TiterMax Gold; CytRx; Norcross, GA). Fourth injections were given seven days after the third injection. Four days after the fourth injection, spleen cells were fused with myeloma cells using polyethylene glycol 1500 (#783641; Roche Diagnostics GmbH; Penzberg, Germany). The fused cells were cultured in six 96 well flat-bottomed micro culture plates for 14 days.

### D. Pretreatment of Rice

Rice powder was prepared by grinding from brown *japonica* rice using a millser. Extract of rice was prepared with 20 ml of 0.1 M HCl adding to 2 g rice powder and incubated at 37˚C for 1 hour. To isolate cadmium, 5 ml of extract was applied to the cadmium isolation column (The general environmental technos, Osaka, Japan). After washing column with 5 ml of 0.1 M HCl, The absorbed cadmium was then eluted from the column using 5 ml of 0.05 M $HNO_3$.

### E. IC procedure

For neutralization of colimn eluate and chelation of Cd, 0.02 ml of the eluate was mixed with 0.38 ml of 0.05 M Tris-HCl (pH 7.5), 0.3 μM EDTA. For IC assay, 75 μl of sample solution was loaded to the IC device. After 40 minutes, the red band appeared on the test line of IC device was read by using an IC reader, DiaScan 30-D (Otsuka Electronics, Osaka, Japan).

## III. RESULTS

### A. Development and Confirmation MAbs to Cd-EDTA

Two Mab clone (clones Nx2C3 and So26G8) were obtained through the screening for the thousands of hybridoma by a KinExA automated immunoassay system(6) with Cd-EDTA conjugate as antigen. The antibody produced by clone Nx2C3 showed 50-fold higher affinity to Cd-EDTA than the antibody from So26G8. Values for the dissociation rate constants for antibody-EDTA chelated metal interactions and cross-reactivities of the antibody are summarized in Table I.

TABLE I
Binding of EDTA complexes to Nx2C3 MAb

| EDTA complex | *Kd* value ( $\mu$ M) | cross reactivity (%) |
|---|---|---|
| Cd(II)-EDTA | 0.013 | 100 |
| Cu(II)-EDTA | 0.96 | 2.4 |
| Mn(II)-EDTA | 1.8 | 1.5 |
| Zn(II)-EDTA | 2.3 | 0.97 |
| Fe(III)-EDTA | 42 | 0.062 |
| Mg(II)-EDTA | 236 | 0.026 |
| Ca(II)-EDTA | > 1000 | < 0.001 |
| metal free EDTA | > 5000 | < 0.002 |

### B. Cd isolation column

Although, as shown in TABLE I, antibody Nx2C3 has high specificity to Cd-EDTA, extracted samples from biomaterials usually contain trace Cd and large amount of another metals, which is enough to interfere with antibody-Cd-EDTA interactions and cause false positive in the immunoassay using the antibody. For example, isolation of Cd from extract of rice was need for the immunoassay because zinc, magnesium and manganese in rice extract prevented reaction of the antibody and Cd. The general environmental technos co., ltd. (Osaka, Japan) provided us chelation column, which separates Cd and other metals and we tested it with rice samples consisting of broad range Cd content.

At first, rice samples were grinded and treated with 0.1 M HCl to extract Cd from rice. In the supernatants of the extracts, Cd concentrations were 0-0.1 mg/kg. On the other hand Zn concentrations and Mn concentrations were 1.5-4.0 mg/kg. Further more Mg concentrations were 40-100 mg/kg. We attempted to isolate Cd from the extracts using the chelation column, loading the extracts to the column and eluting Cd with 50 mM HNO3. The individual recovery of Cd from the rice extract is shown in Fig. 1. From 0.02 mg/kg to 0.1 mg/kg, the column could recover approximately total Cd, whereas average recoveries of Zn, Mn and Mg were less than 5.1 % respectively (Fig. 2). These removals of Zn, Mn and Mg seemed to be enough to carry out the immunoassay for trace cadmium in rice sample.

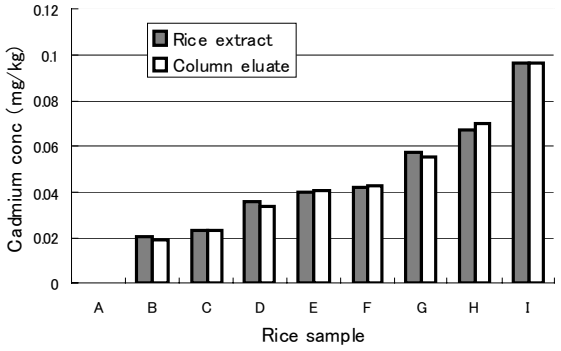


Fig. 1. Recovery test of Cd isolation column. Cd concentration of rice extracts and column eluates were measured using ICP-AES respectively. Cd of sample A was not detected.

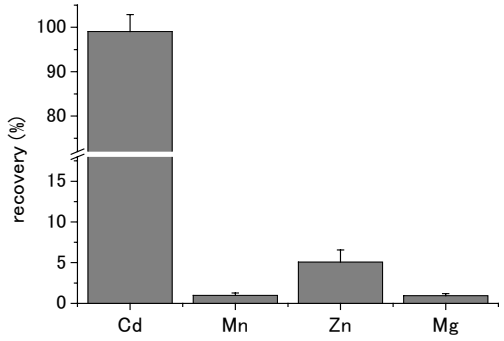


Fig. 2. Recovery of Cd and other major metals in rice. Average recovery for Cd, Mn, Zn and Mg were determined from concentrations of respective concentrations of metals in rice extracts and column eluates from nine rice sample (Fig. 1. A-I) All concentrations were determined by ICP-AES.

### C. Design of immunochromatography format

A format of immunochromatography (IC) for Cd is illustrated in Fig. 3. Before sample loading on the IC device, sample is mixed with EDTA and gold particle labeled anti-CD-EDTA antibody to make complex of them. After loading them, they migrate toward test line where conjugate of Cd-EDTA-OVA is immobilized to capture Cd-EDTA free antibodies. On the other hand, Cd-EDTA free antibodies pass through the test line and are finally captured by anti-Mab antibody at control line. Several minutes after the sample loading, two red bands are observed. One is on the test line, which is decreased proportionately with the cadmium concentration and another one is observed as positive control on the control line.

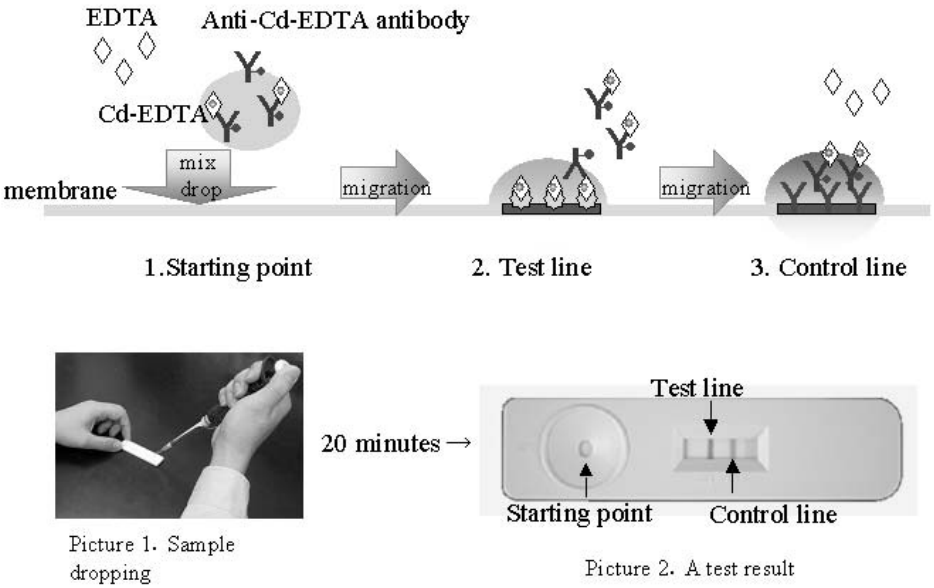


Fig. 3. Principle of the IC.

### D. *Testing of IC*

To profile the red coloring on the test line of the IC device, we tested CdCl2 solution. Fig. 4 shows the results of the measurement using chromatograph-reader for the color digitization of red band appeared on the test line. As the result, the limit of detection of Cd was determined to be 0.3 μg/kg (ppb).

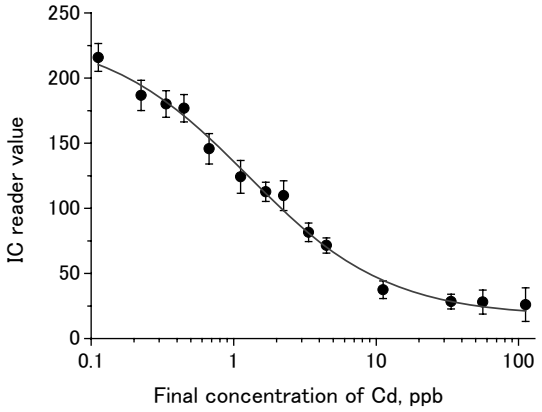


Fig. 4. IC assay of $CdCl_2$.

### E. *Measurement of Cd in brown rice*

About 270 brown rice samples were tested with the Cd isolation column and Cd detection IC. Same samples were also measured using an ICP-AES instrumental analysis. Comparison of these results is shown in Fig. 5. The correlation coefficient (r) was 0.89. In this measurement Cd from rice samples was loaded to IC at 1:200 dilution, which was good for detecting 0.2-0.4 mg/kg Cd in rice. (Codex proposed a maximum level of 0.4 mg/kg (ppm) Cd in rice.)

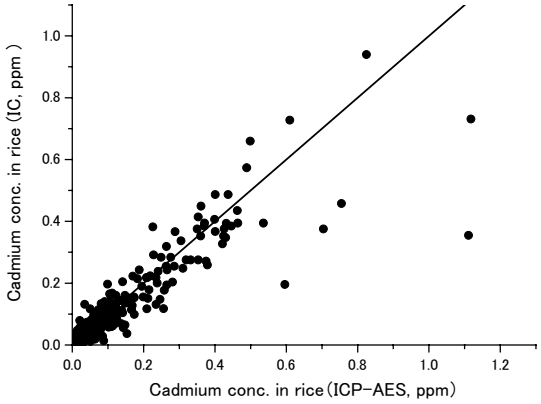


Fig. 5. IC Cd estimate vs. ICP-AES Cd estimate. ICP-AES was carried out with rice extracts and IC was carried out with column eluates.

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