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# Development of dioxin toxicity evaluation method in human milk by enzyme-linked immunosorbent assay—assay validation for human milk

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**Abstract**

In this study, the development of a toxicity evaluation method for dioxins in human milk by enzyme-linked immunosorbent assay (ELISA) was reported. A total of 17 human milk samples were tested by ELISA and by gas chromatography/mass spectrometry (GC/MS) to assess whether the ELISA performed on samples obtained from primiparas could be considered as reliable enough for identifying a dioxins contamination in human milk. The concept of toxicity equivalent quantity (TEQ) screening was validated by comparing TEQ values for a set of human milk samples to the ELISA responses predicted for those samples. A fairly good correlation ($r = 0.920$) between immunoassay and GC/MS was achieved for human milk. This ELISA should be useful for biological samples monitoring. © 2002 Elsevier Science Ltd. All rights reserved.

*Keywords:* TCDD; Immunoassay; Validation; PCDDs; PCDFs; GC/MS

## 1. Introduction

Effective dioxin monitoring requires the analysis of great many samples. However, a traditional method using high-resolution gas chromatography/mass spectrometry (HR-GC/MS) requires a complicated sample cleanup, special equipment and a highly trained analyst. This analytical technique is expensive and time consuming (Schecter, 1998). In spite of a great demand for monitoring dioxins from the government and the public, the cost and time required for analysis often severely limits the scope and thoroughness of a sampling effort. One of the methods that may satisfy the requirements is an enzyme-linked immunosorbent assay (ELISA), and there have been some reports on measurement of dioxins by use of ELISA. However, most of the reports dealt with standard substances (Carlson and Harrison, 1998), fly ash (Zennegg et al., 1998), soil (Harrison and Carlson, 1998, 1999) and chimney soot (Zennegg and Shmid, 1999) which contained dioxins in high concentration. There has been no report on a practical assay that can deal with biological samples containing dioxins in extremely low concentrations such as human milk and blood. The conventional ELISA has been considered as a simple screening method, and to be less reliable compared with GC/MS method. We have developed a polyclonal antibody-based immunoassay for chlorinated

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dibenzo-*p*-dioxins and dibenzofurans (Sugawara et al., 1998). We describe here the application of our immunoassay to human milk with its potential use as a new evaluation method for toxicity.

## 2. Materials and methods

### 2.1. Chemicals and immunoreagents

The surrogate standard for ELISA, 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) was synthesized by Sanborn et al. (1998). Preliminary data indicated these compound responds similarly to 2,3,7,8-TCDD in an antiserum-based ELISA (Sugawara et al., 1998). A novel coating hapten III for 2,3,7,8-TCDD (7,8-dichlorobenzo[5,6][1,4]dioxino[2,3-b]pyridine-3-carboxylic acid) was coupled to bovine serum albumin (BSA). Antiserum #7598 was raised against an immunogen hapten I (5-[3,7,8-trichlorodibenzo-*p*-dioxin-2-yl]-*trans*, *trans*-penta-2,4-dienoic acid). Goat anti-rabbit antibody coupled to horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co.

All dioxin standards for GC/MS were obtained from Wellington Laboratories (USA) and were diluted with special quality grade decane. Most of organic solvents such as hexane, acetone, dichloromethane, toluene, diethylether and ethanol with dioxin analysis grade were purchased from Kanto Kagaku (Tokyo, Japan) or Wako Pure Chemicals (Osaka, Japan). Silica-gel impregnated activated carbon, 44% $H_2SO_4$/silica-gel and silica-gel S-1 were obtained from Wako Pure Chemicals. A three-layer $H_2SO_4$/silica-gel column packed in a disposable cartridge tube (inside diameter 15 mm $\times$ length 75 mm; made of polypropylene) was made by a special request to Supelco (USA).

Three-layer $H_2SO_4$/silica-gel column: silica-gel 1 g, 44% $H_2SO_4$/silica-gel 2 g, silica-gel 0.5 g, and $Na_2SO_4$ 1 g were sequentially accumulated from the lower layer into a glass column having an inside diameter of 1 cm by slurry packing with hexane.

### 2.2. Validation for human milk samples

Human milk samples were collected from Japanese primiparas whose mean age was 28.5 years old. Human milk was sampled on 30 days after birth. These samples were tested by the ELISA.

### 2.3. Sample cleanup

A human milk sample was divided into two aliquots. One was used for the ELISA. Another aliquot was treated by the conventional clean up method and applied to the GC/MS. Fig. 1 shows a flow chart for sample cleanup. Human milk samples were routinely prepared using nonpolar solvents that were incompatible with the ELISA. For ELISA analysis, the sample was passed through the three-layer column after the alkali decomposition step. The eluent was evaporated to dryness under nitrogen and then re-dissolved into 60 μl of MeOH–DMSO (1:1) with 100 ppm Triton X-100 with 5-min sonication and analyzed by ELISA.

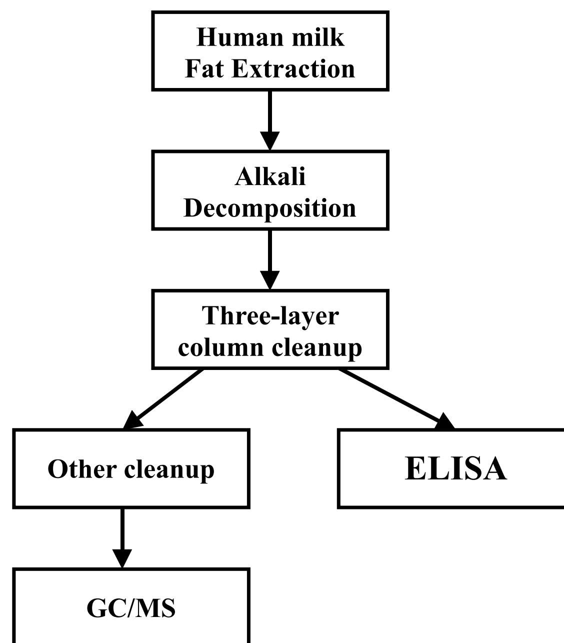


Fig. 1. Flow chart for sample cleanup. Three-layer silica-gel column was used instead of the conventional multi-layer silica-gel column for the cleanup after alkali decomposition. Then the eluent was employed for the ELISA without other cleanup step.

Fat was extracted from human milk samples. After internal standards were added, the fat was stirred with 40 ml of 1 N KOH/ethanol and then stayed overnight at room temperature to carry out alkali decomposition. The solution was diluted with 40 ml of water and followed by liquid–liquid extraction with $2 \times 40$ ml of hexane. The hexane extracts were dehydrated and concentrated. Then, the extracts were cleaned up with a three-layer $H_2SO_4$/silica-gel column with 40 ml of hexane as eluent. The eluate was divided equally into two aliquots; one was for ELISA and another was for GC/MS analysis. The eluate for ELISA was dried out by nitrogen stream, and the residue was re-dissolved into 60 μl of MeOH–DMSO (1:1) with 100 ppm Triton X-100. Another eluate for the GC/MS analysis was further purified with activated carbon silica-gel column chromatography then applied GC/MS.

### *2.4. ELISA*

Microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4 °C with 100 μl/well of the appropriate coating antigen concentration in 0.1 M carbonate–bicarbonate buffer (pH 9.6). Standards were prepared in 1:1:2 (*v*:*v*:*v*) DMSO:MeOH with 100 ppm Triton X-100: phosphate buffered saline (PBS, pH 7.5) containing 2 mg/ml BSA. After an initial blocking step with BSA–PBS, and a wash step, 50 μl of standards were added. Sample wells contained 25 μl of PBSB then 25 μl of human milk sample in DMSO–MeOH was added. Next, 50 μl of the antiserum diluted in PBSB was added to each well. The final ratio of DMSO–MeOH to PBSB was 1:3. The plates were incubated for 90 min. Following a wash step, 100 μl of goat anti-rabbit antibody coupled to horseradish peroxidase was added (diluted in PBS + 0.05% Tween 20). After a 60-min incubation period, the plates were washed with wash buffer, and 100 μl of enzyme substrate containing TMB was added to each well. After 20 min, the color reaction was stopped by addition of 50 μl of 2 M sulfuric acid. The resultant color was measured at 450 nm with a Model 550 Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA) in single-wavelength mode, and dioxin levels in the human milk samples were calculated on the basis of a standard curve derived from a fit of absorbance versus the logarithm of concentration.

### *2.5. GC/MS*

GC/MS was carried out using a JEOL JMS-700 MStation mass spectrometer coupled to a HP-6890 HR-GC with a capillary column of DB-17HT (30 m × 0.25 mm i.d., film thickness 0.15 μm; J&W Scientific) for the isomer specific separation. The GC program was as follows: 150 °C for 1 min, raised 20 °C/min to 220 °C and subsequently 4 °C/min to 280 °C, maintained for 11.5 min. Helium was used as carrier gas. The injector temperature was 280 °C and the GC/MS interface temperature was held at 280 °C. The MS was operated in the selected ion monitoring mode with mass resolution of 10 000, and the electron impact ionization energy was 38 eV, with an ion source temperature of 260 °C. Quantification was done by the isotopic dilution method, i.e. PCDD/Fs congeners were quantitated by comparison with their respective reference $^{13}C_{12}$-labeled standards in the following two ways; in one way, one kind of stable isotope in each congener of PCDD/Fs was used as the internal standard, and in the other way, $^{13}C_{12}$-1,2,3,4-TCDF was for tetra to hexa-CDD/Fs, and $^{13}C_{12}$-OCDD for hepta to octa-CDD/Fs, and then the PCDD/Fs concentrations were calculated by fat basis. The toxicity equivalent quantity (TEQ) was calculated using WHO-TEF (1998).

## 3. Results and discussion

### *3.1. ELISA methods*

Table 1 shows the structures of target analyte TCDD, the surrogate standard for ELISA, TMDD, and dioxin haptens. In the indirect competitive ELISA, the sensitivity is determined by the affinities of two competitive components (coating antigen and target analyte) for the antibody. To obtain greater sensitivity, a new coating antigen hapten should have a lower affinity than that previously reported. A new coating antigen hapten III (Table 1) was designed and synthesized (Shan et al., 2001). As Shan reported, the substitution of N for C in one of the rings made a significant improvement in the ability of TCDD to displace the hapten's binding to the antibody, resulting in a more sensitive assay. The optimized ELISA for human milk samples used the coating antigen III-BSA at a dilution of 1:15 000, the antiserum #7598 at a dilution of 1:3500 and the analyte in DMSO–MeOH–PBSB buffer (1:1:2). The average $IC_{50}$ of 10 standard curves was $11.4 \pm 2.4$ pg/well ($227 \pm 48$ ppt) with a limit of detection (LOD) of $1.1 \pm 0.4$ pg/well ($22 \pm 8$ ppt (Fig. 2)). The LOD was calculated based on the $IC_{10}$ value as reported by Brady (1995). The new coating antigen system also had background values 5-fold lower than the previously reported system and two-times lower LOD.

Table 1
Structures of TCDD, surrogate standard TMDD and dioxin haptens

| Compound | Structure |
|---|---|
| TMDD | Cl, Cl, O, O, $CH_3$, Cl |
| TCDD | Cl, Cl, O, O, Cl, Cl |
| Hapten I | Cl, Cl, O, O, Cl, O, OH |
| Hapten II | Cl, Cl, O, O, H, O, OH |
| Hapten III | Cl, Cl, O, O, N, H, O, OH |

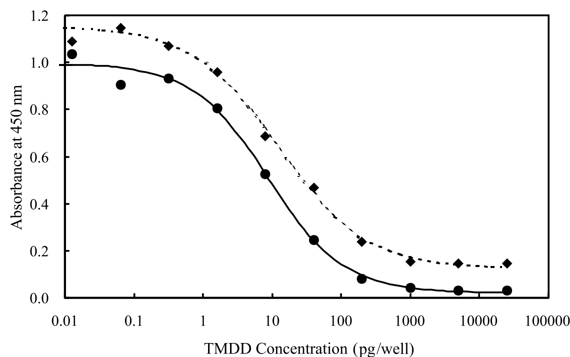


Fig. 2. Comparison between the old standard curve with hapten II(◆) and the new one with hapten III(●). The sensitivity of the new standard curve: $IC_{50} = 11.4 \pm 2.4$ pg/well ($227 \pm 48$ ng/l), LOD $= 1.1 \pm 0.4$ pg/well ($22 \pm 8$ ng/l).

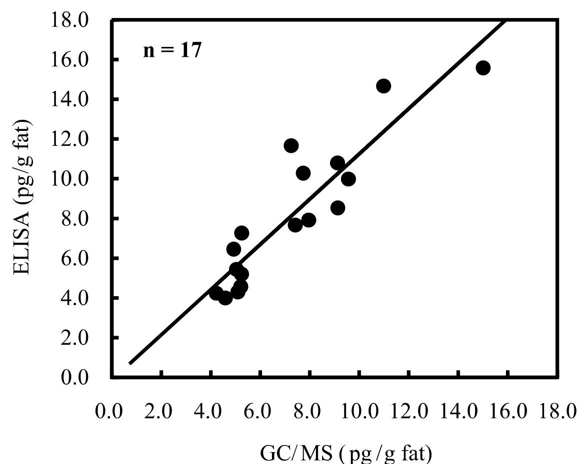


Fig. 3. Relationship between TMDD equivalents by ELISA and GC/MS values. The regression equation for predicted ELISA against GC/MS values was $y = 1.139x - 0.142$ and the correlation coefficient was 0.909. The ELISA values are plotted as a function of the totals of the two most toxic congeners (2,3,7,8-TCDD and 1,2,3,7,8-pentaCDD) as determined by GC/MS.

### *3.2. Milk matrix effects and sample cleanup*

To simplify a sample cleanup, after alkali decomposition, DMSO–MeOH (1:1) was added to the residue from the evaporated hexane extract and then applied to the ELISA. However, a strong matrix effect resulted in fairly high values, compared to the corresponding GC/MS values. Whereas with the new three-layer sulfuric acid silica-gel column cleanup step instead of the conventional multi-layer silica-gel column step, there was no significant matrix effect. As a result, ELISA values showed good agreement with GC/MS results for all samples. Thus these values suggest that this ELISA can rapidly screen and predict dioxins in human milk with only the three-layer sulfuric acid silica-gel column cleanup step and this new column can be used for ELISA as well as for GC/MS simultaneously.

### *3.3. Assay validation*

The results we obtained for the measurement of dioxins in human milk samples are highly encouraging. This work represents the first time that ELISA for dioxins have been run on biologically complex samples with low dioxin concentration.

There are several ways of quantitatively comparing the results of the GC/MS analysis with those obtained by the ELISA. Fig. 3 shows the relationship between TMDD equivalents by ELISA and GC/MS. The ELISA values are plotted as a function of the totals of the two most toxic congeners (2,3,7,8-TCDD and 1,2,3,7,8-pentaCDD) as determined by GC/MS. This ELISA recognized the most strongly these two congeners, whereas it displayed moderate cross-reactivity to 2,3,7,8-TCDF and 2,3,4,7,8-pentaCDF and little or no cross-reactivities to Co-PCBs (Shan et al., 2001). Most of the dioxin levels in human milk we used were in two orders of magnitude of contamination level. Thus to compare the two methods for human milk samples, a linear–linear plot was required to accommodate the narrow range. This is the challenge because chemical contaminants in human milk have almost some narrow range (Darbre, 1998), however the plots previously reported to compare the two methods were almost log–log plots to accommodate wide contamination level of environmental samples (Harrison and Carlson, 1998; Zennegg et al., 1998). An excellent agreement between GC/MS values and ELISA values was obtained from linear regression analysis ($y = 1.139x - 0.142$, $r = 0.909$, $n = 17$). The ELISA should identify each sample as having at least as much contamination as represented by the sum of the most toxic two congeners, so all of the data points should be on or above the line of identity. Indicating the slope was 1.1, the ELISA gave slightly higher than the GC/MS values. These results indicate that this ELISA should be suitable for screening samples prior to GC/MS. A positive result of the ELISA would indicate contamination. Human milk samples identified as positive by the ELISA screening should then be subjected to confirmatory GC/MS analysis to establish the revels of individual congeners present.

Another way of plotting the data is shown in Fig. 4, where the totals of TEQ (PCDD/F) determined by GC/MS are plotted against the ELISA estimates of contamination. A fairly good correlation between ELISA values and TEQ (PCDD/F) values was also observed with human milk. The TEQs were calculated based on WHO-TEF (Van den Berg et al., 1998).

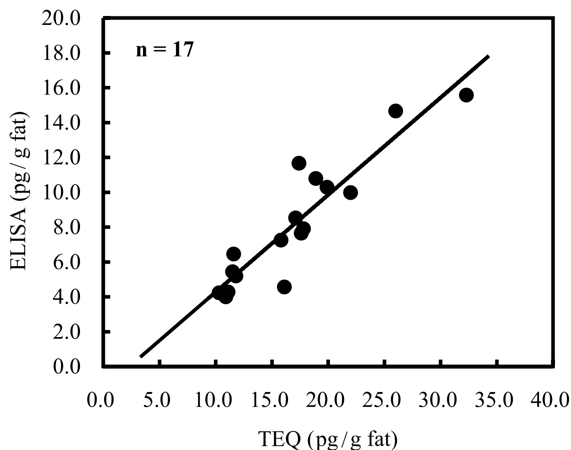


Fig. 4. Relationship between TMDD equivalents by ELISA and TEQ (PCDD/F) values. The regression equation for predicted ELISA against TEQ was $y = 0.558x - 1.301$ and the correlation coefficient was 0.920. Values for individual congener concentrations from GC/MS analysis were used to calculate TEQ values.

Since ELISA has a property of cross-reactivity, it cannot determine each of different congeners unlike GC/MS method. However, by use of an antibody that has high reactivity to congeners with a high toxicity but poor reactivity to congeners with a low toxicity, the ELISA provides not only the relative TCDD equivalent values recognized by the antibody but also degrees of toxicity that is predicted to be directly related to the toxic potency of the complex mixture.

The slope value of the linear regression equation was less than 1 (0.558). Because more than 70% of TEQ in PCDD/F consisted of TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF, it is reasonable that a moderate cross-reactivity to 2,3,4,7,8-PeCDF influenced the underestimation of ELISA values. However, a strong correlation ($r = 0.920$) and the linear regression equation between ELISA and TEQ suggest that multiplying ELISA values by the reciprocal number of the slope value (1.8) might give practical estimation for dioxins in human milk samples without the benefit of GC/MS analysis. One of the main areas addressed by this study was pointing out that the ELISA could measure a low concentration of dioxins in biological samples. Although the more plots are required, if the reciprocal number of the slope is used a function of dioxin contamination of various human milk samples, the initial results from our application of the ELISA assay to human milk samples suggest usefulness as a toxicity evaluation method for dioxins in human milk. For it has been known that Co-PCBs have the toxicity like dioxins, current TEQ value in a sample is evaluated the sum of TEQ value by PCDD/Fs and that by Co-PCBs. Thus current ELISA for dioxin contaminants might be required to recognize the Co-PCBs in the analysis. If only knowing the degree of toxicity of a sample without a specification of the cause-materials is required as an dioxin analysis, the useful bioassays have already reported like aryl hydrocarbon receptor (Ah-receptor) methods (Safe et al., 1989). However, it is one of the most important factors for an analysis to specify the cause-materials, even if the analysis is an analysis for the toxicity evaluation. There is the specificity to some specific chemicals to antibodies reaction but not to Ah-receptor reaction. Hence although the ELISA could be improved on the weak recongnition to the Co-PCBs, it has high usefulness to detect the toxicity of PCDD/Fs. To improve to detect the total toxicity of dioxin family including Co-PCBs, additional antibiodies could be selected that will meet to cover the detection of the Co-PCBs. If excellent anti-Co-PCBs antibodies were available, they could be used with the current antibodies in an assay. A panel of antibodies could provide congener profiles for samples similar to the profiles now obtained with GC/MS.

## 4. Conclusion

Enzyme immunoassay is a very practical screening tool for the determining trace levels of dioxin in human milk. A good correlation between the ELISA values and TEQ values for human milk sample indicates that this assay can be used as a TEQ screening method for PCDDs and PCDFs. The sample preparation method with a three-layer column was easy to perform and used as GC/MS-compatible preparation method for PCDD/Fs in human milk. This work demonstrates the practical use of the ELISA for a new evaluation method for toxicity. The development of the GC/MS-compatible preparation method for PCDD/Fs in environmental matrices will make the ELISA a powerful tool for these analyses.

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