Development of Dioxin Toxicity Evaluation Method in Human Milk by Enzyme-Linked Immunosorbent Assay (part III: Assay Validation for Human Milk)

Yukio Sugawara[1], Koichi Saito[2], Masahiko Ogawa[2], Susumu Kobayashi[2], Guomin Shan[3], Bruce D. Hammock[3], Hiroyuki Nakazawa[4] and Yasuhiko Matsuki[5*]

[1]R & D Planning Group, Cosmo Research Institute, 4-9-25, Shibaura, Minato-ku, Tokyo 108-8564, Japan
[2]Dioxin Research Group, Saitama Institute of Public Health, 639-1, Kamiokubo, Urawa, Saitama 338-0824, Japan
[3]Department of Entomology, University of California, Davis, CA 95616, U.S.A.
[4]Department of Analytical Chemistry, Hoshi University, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan
[5] Food and Drug Safety Center, Hatano Research Institute, 729-5, Ochiai, Hadano, Kanagawa 257-8523, Japan

Introduction

Effective dioxin monitoring requires the analysis of a great many samples. However, a traditional method using high-resolution gas chromatography/mass spectrometry (HR-GC/MS) requires a complicated sample cleanup, specialized equipment and a highly trained analyst. This analytical technique is expensive and time consuming, and worldwide there are few qualified laboratories that can perform these analyses[1]. 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. Immunoassays can be rapid, inexpensive and quantitative with detection limits in the low picogram level. We have developed polyclonal antibody-based immunoassay that react with chlorinated dibenzodioxins and dibenzofurans[2]. We describe here the application of our immunoassay to human milk with its potential use for a new evaluation method for toxicity.

Methods and Materials

1. Chemicals and Immunoreagents

Surrogate standard for ELISA 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) was synthesized in Dr. Hammock's laboratory[3]. Preliminary data indicated this compound responds similarly to TCDD in an antiserum based ELISA[2]. Two different coating haptens for TCDD were coupled to Bovine Serum Albumin (BSA). Antiserum #7598 was raised against an immunogen hapten I. Goat anti-rabbit antibody coupled to horseradish peroxidase and 3,3',5,5'-tetra-methylbenzidine (TMB) were purchased from Sigma Chemical Co. The instruments and the conditions were described in Part II.

2. Sample Cleanup

Human milk was split into two aliquots. One of them was cleaned up by the same cleanup method

in Part II till the three-layer sulfuric acid silica gel column cleanup (three-layer column cleanup) step. The eluent was evaporated to dryness under nitrogen and then redissolved into 60 μL of MeOH-DMSO (1:1) with 100 ppm Triton X-100 for ELISA under 5-min sonication and applied for ELISA.

3. ELISA

The ELISA method previously reported was modified by using a new coating antigen (Hapten III)[2]. Microtiter plates were coated with coating antigens (hapten-BSA conjugates). 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 bovine serum albumin (BSA) [DMSO:MeOH:PBSB]. After an initial blocking step with BSA-PBS, and a wash step, 50 μL of standards were added. 25 μL of PBSB was added to wells for samples, then 25 μL of human milk sample in DMSO-MeOH was added to the above samples wells. 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 plates were read at 450 nm.

Results and Discussion

1. New coating antigen hapten design

Table 1 shows the structures of target analyte TCDD, surrogate standard for ELISA TMDD and dioxin haptens. In competitive ELISA format, assay sensitivity is determined by the difference of affinities of two competitive components (coating antigen and target analyte) with the antibody. If the antibody has a much lower binding affinity against coating antigen than that against the target analyte, the binding of the coating antigen to the antibody can be easily displaced by a very low amount of the target analyte. Thus to obtain a better sensitivity, a new coating antigen hapten should have a lower affinity than that previously reported. A new coating antigen hapten III in Table 1 was designed and synthesized. Substitution of N for C in the ring makes much difference in the structure between the new coating hapten and the immunizing hapten (hapten I) as well as the target analyte TCDD. The optimized ELISA used the coating antigen III-BSA at a dilution of 1:15000, 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 227 ± 48 ng/L with a lower detection limit (LDL) of 22 ± 8 ng/L. The LDL was calculated as the IC_{10} value[4]. A new coating antigen system contributed to obtain the background values 5-fold lower than the previously reported system but not a significantly lower IC_{50} and LDL.

2. Cross-reactivity

In PCDD, TCDD has the highest cross reactivity (129%) with this assay, and followed by 1,2,3,7,8-pentaCDD (72%). Whereas in PCDF, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF displayed moderate cross-reactivities. According to Saito's work at this time, it was obvious that more than 70% of toxicity equivalents (TEQ) of PCDD/F in human milk consisted of three congeners mainly; TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF, which have 1.0, 1.0 and 0.5 as toxicity equivalent factor (TEF) values respectively. Because this ELISA showed the strong cross-

reactivities to TCDD and 1,2,3,7,8-PeCDD but not to 2,3,4,7,8-PeCDF, it suggests that this ELISA might be a good indicator of toxicity of dioxins in human milk.

3. Milk Matrix Effects and Sample Cleanup

To simplify a sample cleanup, after alkali decomposition, DMSO-MeOH (1:1) was added to hexane extraction dried out by nitrogen stream and then applied to the ELISA. However, it has strong matrix effects that showed fairly high values, compared to the corresponding GC/MS values. Whereas with the new three-layer column cleanup step as described in part II instead of the conventional multi-layer silica gel column step, there were no significant matrix effects. 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 column cleanup step and this new column can be used for ELISA as well as for GC/MS simultaneously.

4. Assay Validation

A good agreement between GC/MS values and ELISA values was obtained from linear regression analysis ($y=1.139x-0.142$, $r=0.909$, $n=17$). A fairly good correlation between ELISA values and TEQ (PCDD/F) values was also observed with human milk (Figure 1). The TEQs were calculated based on WHO-TEF (1998). The slope value of the linear regression quotation was less than 1 (0.558). Because more than70% 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 (1.792) might give practical estimation for dioxins. Thus this ELISA indicated usefulness as a toxicity evaluation method for dioxins in human milk.

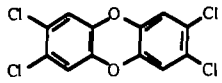
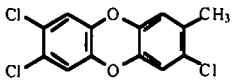
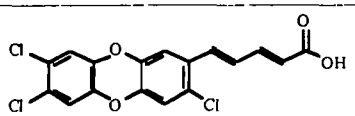
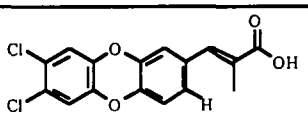
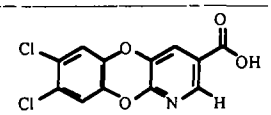
Acknowledgement

This project was supported in part by Health Sciences Research Grants from the Ministry of Health and Welfare of Japan and NIEHS Superfund Research Program, 5 P42 ES04699-08, the NIEHS Center for Environmental Health Sciences and USEPA Center for Ecological Health Research CR819658(U.S.A.).

Reference

1. Schecter, A. (1998) Env. Health_Persp. 106, Suppl 2, 737-742.
2. Sugawara, Y., Gee, S.J., Sanborn, J.R., Gilman, S.D. and Hammock, B.D. (1998) Anal. Chem. 70, 1092-1099.
3.Sanborn, J.R., Gee, S.J., Gilman, S.D., Sugawara, Y., Jones, A.D., Rogers, J., Szurdoki, F., Stanker, L.H., Stoutamire, D.W. and Hammock, B.D. (1998) J. Agric. Food Chem. 46, 2407-2416.
4. Brady, J.F. (1995) in: Immunoanalysis of Agrochemicals (Karu, A.E., Nelson, J.O. and Wong, R.B., Ed.), American Chemical Society, ISBN 0-8412-3149-4

Table 1. Strucures of TCDD, surogate standard TMDD and Dioxin Haptens

Compound	Structure
TCDD	
TMDD	
Hapten I	
Hapten II	
Hapten III	

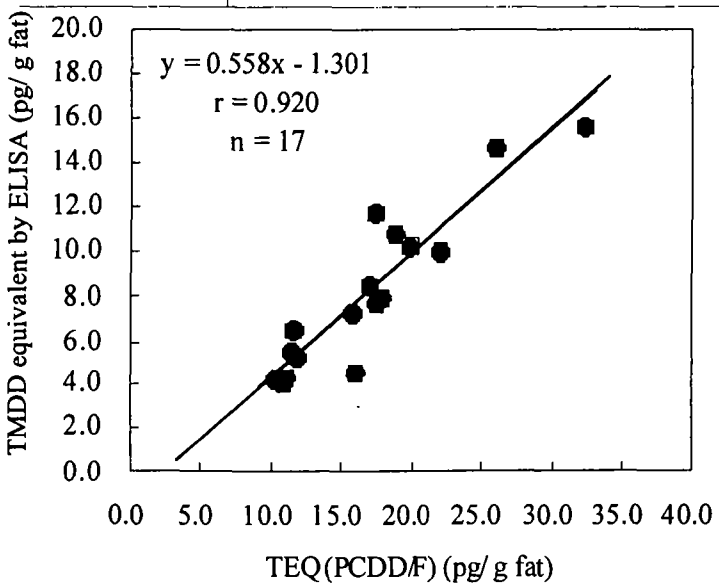


Figure 1. Relationship between TMDD equivalent by ELISA and TEQ (PCDD/F) values calculated upon GC/MS