*Anal. Chem.* **1998,** *70,* 1092−1099

# Development of a Highly Sensitive Enzyme-Linked Immunosorbent Assay Based on Polyclonal Antibodies for the Detection of Polychlorinated Dibenzo-*p*-dioxins

Yukio Sugawara,[†] Shirley J. Gee, James R. Sanborn, S. Douglass Gilman,[‡] and Bruce D. Hammock*

*Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616*

**The development of an enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibodies for the polychlorinated dibenzo-*p*-dioxins is described. We previously reported the synthesis of haptens and generation of antibodies for detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Antisera were screened with seven different coating antigens (hapten−protein conjugates), including *trans*-3-(7,8-dichlorodibenzo-*p*-dioxin-2-yl)-*cis*-2-methylpropenoic acid (VII) and 5-(3,7,8-trichlorodibenzo-*p*-dioxin-2-yl)penta-*trans*,*trans*-2,4-dienoic acid (X). All inhibition screening and optimization studies were conducted using a less toxic surrogate standard for TCDD [2,3,7-trichloro-8-methyl-dibenzo-*p*-dioxin (TMDD; XVII)] which responded similarly to 2,3,7,8-TCDD in the ELISA. The most sensitive assay from the screening studies [coating antigen VII-BSA, 0.1 μg/mL, and antiserum 7598 (anti-X-LPH), 1:10000] was further optimized and characterized. It exhibited an $IC_{50}$ value of 12 pg/well (240 pg/mL), with working range from 2 to 240 pg/well (40 to 4800 pg/mL). The influence of various physical and chemical factors (time, solvent, detergent) was investigated. The optimized assay was then used to assess cross-reactivity by congeners of halogenated dioxins and related structures. DMSO up to concentrations of 37.5% decreased the $IC_{50}$ value in the assay, whereas methanol to concentrations of 30% did not lead to improved $IC_{50}$ values.**

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are a well-known group of highly toxic and widespread environmental pollutants. They are found in commercial chlorophenols and their derived products, emissions from incineration sources, and byproducts from pulp and paper mills.[1] PCDDs and PCDFs have been found in many kinds of environmental matrixes including air, soil, sediment, fish and human adipose tissue, and milk.[1,2] Because they are highly toxic and persistent, regulatory agencies have investigated the potential adverse human health effects and environmental damage due to these compounds.[3−6] PCDDs have 75 positional congeners and there is a wide difference in toxicity among them.[1,7] In particular, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is known as the most toxic congener. The $LD_{50}$ value of this compound is 0.6−2.0 μg/kg in guinea pigs.[8] Because the toxicity varies by congeners, the analyses for PCDDs require the identification and quantification of each isomer and congener.

In spite of the large need to monitor PCDDs and PCDFs, the only analytical technique with sufficient sensitivity (parts per trillion) and selectivity for determination of PCDDs including 2,3,7,8-TCDD is a combination of high-resolution gas chromatography and high-resolution mass spectrometry.[9] This analytical technique is expensive, and it requires specialized equipment, a highly trained analyst, and a dedicated laboratory. Depending on the amount of sample preparation needed, the analysis can take several days to complete. As a result, the screening of large numbers of samples has been limited[10] and supplemental methods are in demand. Ideally these methods would be sensitive, rapid, cost-effective, field-portable, and specific for the most toxic dioxin congeners.

Immunoassays satisfy many of these criteria and have a proven record in detecting clinically significant substances such as cancer markers in clinical diagnostics.[11] Since the potential of immunoassays for pesticide analysis has been reported, many immu-

* Corresponding author: (e-mail) bdhammock@ucdavis.edu; (Fax) 530-752-1537.

[†] Current address: Cosmo Research Institute, 1134-2, Gongendo, Satte-shi, Saitama 340-0193, Japan.

[‡] Current address: Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600.

(1) Safe, S.; Hutzinger, O. In *Polychlorinated Dibenzo-p-dioxins and -furans (PCDDs/PCDFs): Sources and Environmental Impact, Epidemiology, Mechanisms of Action, Health Risks*; Safe, S., Hutzinger, O., Hill, T. A., Eds.; Environmental Toxin Series 3; Springer-Verlag: New York, 1990; pp 1−22.
(2) Sakurai, T.; Kim, J.-G.; Suzuki, N.; Nakanishi, J. *Chemosphere* **1996**, *33*, 2007−2020.
(3) Poland, A.; Knutson, J. C. *Annu. Rev. Pharmacol. Toxicol.* **1982**, *22*, 517−554.
(4) Kimbrough, R. D.; Falk, H.; Stehr, P.; Fries, G. *J. Toxicol. Environ. Health* **1984**, *14*, 47−93.
(5) Muto, H.; Takizawa, Y. *Bull. Environ. Contam. Toxicol.* **1992**, *49*, 701−707.
(6) Giesy, J. P.; Ludwig, J. P.; Tillitt, D. E. *Environ. Sci. Toxicol.* **1994**, *28*, 128A−135A.
(7) Rappe, C. *Environ. Sci. Technol.* **1984**, *18*, 78A−90A.
(8) Kociba, R. J.; Schwetz, B. A. *Drug Metab. Rev.* **1982**, *13*, 387−406.
(9) USEPA; Method 1613: Tetra- through Octa Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HPMS, Revision B; USEPA Office of Water Regulations and Standard, 1994.
(10) Crummett, W. B.; Lamparski, L. L.; Nestrick, T. J. *Toxicol. Environ. Chem.* **1986**, *12*, 111−135.
(11) Schwartz, M. K. *Scand. J. Clin. Lab. Invest.* **1995**, *55*, 5−14.



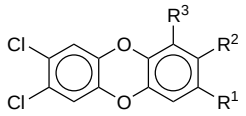
S0003-2700(97)00820-2 CCC: $15.00 
Published on Web 02/10/1998

noassays have been developed for environmental monitoring of pesticides.[12,13] Several government and nongovernment organizations in the United States (EPA, USDA, AEIC, AOAC) and Europe (German Immunoassay Study Group, UK ESCA) are currently involved in the evaluation of immunoassays and the proposal of guidelines for their use as approved analytical methods.[13] The sensitivity, selectivity, precision, and ability to measure analytes without rigorous sample preparation make immunoassays highly cost-effective methods for routine analyses in clinical fields. These advantages also make immunoassays excellent tools for screening of large numbers of samples for environmental monitoring.

The application of immunoassays to the analysis of the halogenated biphenyls, chlorinated hydrocarbon insecticides, halogenated dibenzo-*p*-dioxins, and dibenzofurans has not been as frequent as for more water-soluble species.[13] Since immunoassays typically are aqueous-based systems, the low water solubility of these compounds makes the use of immunoassays more challenging. Attempts to detect PCDDs by immunoassays have been reported.[14,15] The reported radioimmunoassay (RIA) was time-consuming and utilized polyclonal antibodies (PAbs).[14] Monoclonal antibodies (MAbs) developed by Kennel et al.[15] lacked selectivity for free dioxin in solution. Stanker et al. generated MAbs to dioxin and developed MAb-based enzyme-linked immunosorbent assays (ELISA).[16−19] The selectivity of the ELISA was very similar to that of the RIA. The optimized assay detected 200 pg/well 2,3,7,8-TCDD as the $IC_{50}$ (the analyte concentration giving 50% inhibition).[19] Langley et al.[20] reported the development of PAb-based ELISAs that detected 1 ng/well 2,3,7,8-TCDD as the $IC_{50}$. Recently, Harrison and Carlson[21] developed a tube test and a microplate test using one of Stanker's MAbs, and the two formats displayed detection limits of 100 and 25 pg/well 2,3,7,8-TCDD, respectively. While these results have led to increased sensitivity, further improvements are needed to approach the detection limits of GC/MS techniques (1 pg of 2,3,7,8-TCDD or less in a 1-g sample).[7]

In this study, we report the development of a highly sensitive ELISA for PCDDs with new polyclonal antibodies. The influence of various physical and chemical factors (time, solvent, detergent) was investigated. The optimized assay was then used to assess cross-reactivity by congeners of halogenated dioxins and related structures. Furthermore, because of the toxicity and the high cost of disposal of 2,3,7,8-TCDD, the use of 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD; **XVII**) as a surrogate standard in the ELISA was investigated.

Table 1. Structures of Dioxin Haptens and Surrogate Standards




| compd no. | R1 | R2 | R3 |
|---|---|---|---|
| 2,3,7,8-TCDD | Cl | Cl | H |
| **IV** | Cl | Cl | CH=CHCOOH |
| **VII** | H | $CH{=}C(CH_3)COOH$ | H |
| **X** | Cl | $(CH{=}CH)_2COOH$ | H |
| **XI** | Cl | CH=CHCOOH | H |
| **XII** | H | CH=CHCOOH | H |
| **XIII** | Cl | $CH{=}CHC_6H_4COOH$ | H |
| **XVI** | Cl | $CH{=}CHC_6H_4NH_2$ | H |
| **XVII** | Cl | $CH_3$ | H |
| **XVIII** | H | $CH_3$ | H |

## MATERIALS AND METHODS

**Chemicals.** The standards 2,3,7,8-TCDD, 1,2,3,4-TCDD, 1-chlorodibenzo-*p*-dioxin (1-CDD), 2,7-dichlorodibenzo-*p*-dioxin (DiCDD), and 3,3′,4,4′-tetrachlorobiphenyl (TCB) were purchased from Chem Service (West Chester, PA). 1,3,7,8-TCDD, 2,3,7-trichlorodibenzo-*p*-dioxin (TriCDD), 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HexaCDD), octachlorodibenzo-*p*-dioxin (OCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PentaCDD), 2,3,4,7,8-PentaCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF), and 2,3,4,7,8-pentachlorodibenzofuran (PentaCDF) were purchased from Cambridge Isotope Laboratories (Andover, MA). Bovine serum albumin (BSA), hemocyanin from *Limulus polyphemus* (LPH), goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate, Tween 20, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemical reagents were purchased from Fisher Scientific (Pittsburgh, PA), or Aldrich Chemical Co., Inc. (Milwaukee, WI).

Surrogate standards, TMDD (**XVII**) and 2,3-dichloro-7−methyldibenzo-*p*-dioxin (DMDD; **XVIII**) and the haptens used in this study were synthesized and described by Sanborn et al.[22] Structures are shown in Table 1. Carboxylic acid haptens were coupled by a mixed-anhydride method and the amine haptens by diazotization as previously reported.[22] Antibodies were generated to compounds **IV**, **X**, **XI**, **XII**, and **XIII** coupled to LPH. Coating antigens were prepared by coupling haptens (**IV**, **VII**, **X**, **XI**, **XII**, **XIII**) to BSA.[22]

**Instruments.** Absorbances for ELISAs were measured with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm). The inhibition curves were analyzed with a commercial software package (Softmax, Molecular Devices). All of the data presented from the ELISA experiments correspond to the average of quadruplicate wells.

**Screening of Antisera by Two-Dimensional Titration**. For the determination of the initial titers for each antiserum, one

(12) Hammock, B. D.; Mumma, R. O. In *Pesticide Analytical Methodology*, Harvey, J., Jr., Zweig, G., Eds.; ACS Symposium Series 136; ACS Publications: Washington, DC, 1980; pp 321−352.
(13) Meulenberg, E. P.; Mulder, W. H.; Stoks, P. G. *Environ. Sci. Technol.* **1995**, *29*, 553−561.
(14) Albro, P. W.; Luster, M. I.; Chae, K.; Chaudhary, S. K.; Clark, G.; Lawson, L. D.; Corbett, J. T.; McKinney, J. D. *Toxicol. Appl. Pharmacol.* **1979**, *50*, 137−146.
(15) Kennel, S. J.; Jason, C.; Albro, P. W.; Mason, G.; Safe, S. H. *Toxicol. Appl. Pharmacol.* **1986**, *82*, 256−263.
(16) Stanker, L.; Watkins, B.; Vanderlaan, M.; Budde, W. L. *Chemosphere* **1987**, *16*, 1635−1639.
(17) Stanker, L. H.; Watkins, B.; Rogers, N.; Vanderlaan, M. *Toxicology* **1987**, *45*, 229−243.
(18) Vanderlaan, M.; Stanker, L. H.; Watkins, B. E. *Environ. Toxicol. Chem.* **1988**, *7*, 859−870.
(19) Watkins, B. E.; Stanker, L. H.; Vanderlaan, M. *Chemosphere* **1989**, *19*, 267−270.
(20) Langley, M. N.; Chopra, R. K.; Creaser, C. S.; Taylor, R. J. K.; Rose, M. D.; Startin, J. R.; Lee, H. A.; Morgan, M. R. A. *Food Agric. Immunol.* **1992**, *4*, 143−152.
(21) Harrison, R. O.; Carlson, R. E. *Chemosphere* **1997**, *34*, 915−928.
(22) Sanborn, J. R.; Gilman, S. D.; Gee, S. J.; Sugawara, Y.; Jones, A. D.; Rogers, J. M.; Stoutamire, D. W.; Denison, M. S.; Hammock, B. D., submitted to *J. Agric. Food Chem*.

microtiter plate (Nunc, No. 442404, Maxisorp, Roskilde, Denmark) was divided into 16 equal parts (one part: 3 columns, 2 rows) and coated with 100 μL of six different coating antigens per well (**IV**-BSA, **VII**-BSA, **X**-BSA, **XI**-BSA, **XII**-BSA, **XIII**-BSA) at a concentration of 0.1 μg/mL in carbonate−bicarbonate coating buffer (1.59 g/L $Na_2CO_3$, 2.93 g/L $NaHCO_3$, pH 9.6) separately. The plates were sealed with adhesive plate sealers and incubated overnight at 4 °C. The following day, the coated plates were washed five times with one-tenth strength phosphate-buffered saline (PBS) with 0.05% (v/v) Tween 20 [0.1× PBST (PBS with Tween 20): 0.8 g/L NaCl, 0.115 g/L $Na_2HPO_4$, 0.02 g/L $KH_2PO_4$, 0.02 g/L KCl, and 0.5 mL/L Tween 20, pH 7.5]. Aliquots of 50 μL/well analyte (0, 10, and 100 μg/L) in 1:2 (v/v) DMSO−PBSTB buffer [normal strength PBSTB (PBST with BSA): 8.0 g/L NaCl, 1.15 g/L $Na_2HPO_4$, 0.2 g/L $KH_2PO_4$, 0.2 g/L KCl, 0.5 mL/L Tween 20, and 2.0 g/L BSA, pH 7.5] and 50 μL/well antiserum diluted in PBSTB were placed on the plates and incubated for 60 min at room temperature. After the plates were washed again with 0.1× PBST to remove any unbound material, 100 μL/well goat anti-rabbit HRP conjugate diluted 1:3000 in PBST was added and incubated for 60 min at room temperature. The plates were washed again, and 100 μL of substrate solution (2.4 mg of TMB dissolved in 400 μL of DMSO and 100 μL of 1% $H_2O_2$ in 25 mL of 0.1 M citrate−acetate buffer, pH 5.5) was added to each well. After 20 min, 50 μL of 2 M sulfuric acid was added to each well to stop the enzyme reaction. The plates were then read in a dual-wavelength mode at 450 − 650 nm.

**ELISA.** The ELISA was conducted using a competitive coated antigen format.[23] Microtiter plates were coated with the optimized concentration (0.1 μg/mL, 100 μL/well) of antigen **VII**-BSA in a carbonate−bicarbonate coating buffer (pH 9.6). They were incubated overnight at 4 °C. The following day, the plates were washed five times with 0.1× PBST and were incubated for 30 min with 300 μL of a 0.5% (w/v) BSA in PBS (blocking solution) per well. The plates were washed again and 50 μL/well analyte in (1:1) DMSO/PBSB buffer (PBSB; PBS with 2.0 g/L BSA) and 50 μL/well antiserum diluted in PBSB (1:5000) were placed and incubated for 90 min at room temperature. The final ratio of DMSO to PBSB was 1:3, and the final concentration of antiserum was 1:10000 in the wells. After another washing step, 100 μL/well goat anti-rabbit HRP conjugate (1:3000) in PBST was added and incubated for 60 min at room temperature. The plates were washed again, 100 μL/well substrate solution was added, and after 20 min, the enzyme reaction was stopped by addition of 50 μL/well 2 M sulfuric acid. The absorbance was read at 450 − 650 nm.

**Preparation of the Standard Curve.** Due to the toxicity of TCDD and the high cost of disposal, all screening studies were conducted using a surrogate standard, TMDD. Preliminary data indicated this compound bound to 2,3,7,8-TCDD in a MAb-based ELISA similar to that reported by Stanker et al.[16,17] This compound, however, is presumed to be less toxic, based on the reports of Romkes et al.[24]

Once a suitable assay system was identified, cross-reactivity with 2,3,7,8-TCDD was verified.

**TMDD** was prepared in DMSO. The stock solutions were serially diluted to 0.0128 pg/well with DMSO−PBSB (1:1), unless otherwise indicated. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentrations. Using the Softmax software package, sigmoidal competitive curves were fitted to a four-parameter logistic equation

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where $A$ is the maximum absorbance at zero concentration, $B$ is the curve slope at the inflection point, $C$ is the $x$ analyte concentration giving 50% inhibition ($IC_{50}$), and $D$ is the minimum absorbance (background signal) at infinite concentration.[25]

**Solvent Effect.** The effect of DMSO was tested by dissolving the analyte in a mixture of PBSTB and DMSO in various proportions (0−100% DMSO). The mixtures were incubated with antiserum in PBSTB on the coated plate. In addition, the following water-soluble organic solvents were tested in the assay: methanol, ethanol, DMF, acetonitrile, ethyl acetate, 2-methoxyethanol, ethylene glycol, and 1,4-dioxane. Concentrations tested were 10, 20, 40, and 60% in PBSTB for a final concentration in the wells of 5, 10, 20, and 30%, respectively (with ethyl acetate tested to the limit of its solubility). The first incubation time was 60 min. Other assay conditions were as described in the ELISA section.

**Time Effect (First and Second Incubations).** Both incubation times in which antisera were added (anti-TCDD antisera, first incubation; goat anti-rabbit IgG−HRP conjugate, second incubation) were tested. Five different first incubation times (60−240 min) and three different second incubation times (20−60 min) were evaluated.

DMSO/PBSTB (1:2) was utilized for the standard preparation. The standard range 7.1−3625 pg/well was utilized for all incubation time tests. Other assay conditions were as described in the ELISA section.

**Effect of Detergent (Tween 20).** To characterize the effect of Tween 20, PBSB, and PBSTB were used to prepare DMSO/PBS(T)B (1:1) buffer for diluting the analyte solution. Following the preparation of the analyte solution, PBSB and PBSTB buffer were also used for dilution of antiserum. Plate washing was conducted with 0.1× PBST as described in the ELISA section.

**Determination of Cross-Reactivities.** The cross-reactivities (CR) were calculated relative to the concentration producing 50% inhibition ($IC_{50}$) by TMDD. The data were obtained from standard curves of the related compounds and calculated according to the following formula:

$$\%\ \mathrm{CR} = (\mathrm{IC}_{50}\ \text{of TMDD}/\mathrm{IC}_{50}\ \text{of the cross-reacting compound}) \times 100$$

**Safety Considerations.** Although every effort was made to avoid exposure to TCDD during the assays, extreme caution is necessary because the toxicity of the compounds utilized in this study is unknown. When dioxins and related compounds are

(23) Voller, A.; Bidwell, D. E.; Bartlett, A. *Bull. W. H. O.* **1976**, *53*, 55−64.
(24) Romkes, M.; Piskoska-Pliszczynka, J.; Key, B.; Safe, S.; Fujita, T. *Cancer Res.* **1987**, *47*, 5108−5111.
(25) Rodbard, D. In *Ligand Assay*; Langan, J., Clapp, J. J., Eds.; Masson Publishing: New York, 1981; pp 45−99.

Table 2. Two-Dimensional Titration Summary for Anti-TCDD Antisera[a]

| | coating antigen | | | | | | |
|---|---|---|---|---|---|---|---|
| rabbit no. | IV−BSA | VII−BSA | X−BSA | XI−BSA | XII−BSA | XIII−BSA | XIV−BSA |
| 69 | | − | − | − | − | + | − |
| 89 | homol[b] | +++[c] | +++ | ++ | ++ | +++ | − |
| 99 | | +++ | + | + | + | ++ | − |
| 7598 | − | +++ | | − | − | + | − |
| 7599 | − | +++ | homol | + | + | ++ | − |
| 7600 | − | +++ | | − | − | + | − |
| 5156 | − | − | − | | − | − | − |
| 5163 | − | − | − | homol | − | − | − |
| 5164 | − | + | − | | − | + | − |
| 2114 | − | ++ | − | − | | ++ | − |
| 2525 | − | − | − | − | homol | − | − |
| 2549 | ++ | +++ | + | ++ | | +++ | − |
| 2492 | + | +++ | + | + | + | | − |
| 2493 | − | − | − | − | − | homol | − |
| 2494 | + | +++ | + | + | + | | − |

[a] The data shown are at a coating antigen concentration of 0.1 $\mu$g/mL and an antibody dilution of 1:10000. [b] homol, homologous system in which the coating hapten is the same as was used for immunization. [c] (−) absorbance <0.25; (+) absorbance 0.25−0.50; (++) absorbance 0.5−0.75; (+++) absorbance >0.75.

handled, two pairs of protective gloves should be worn with some water between the two layers to avoid penetration of highly lipophilic compounds. Activated carbon can be used to eliminate TCDD-like substances from waste solutions. Wearing a laboratory coat and a pair of safety glasses to protect yourself from unexpected spills is also recommended. UV light has been reported to degrade TCDD and some related compounds and, thus, may be useful for cleanup.[26,27]

## RESULTS AND DISCUSSION

**Screening for Titer.** Titers for 15 antisera using 6 different coating antigens are shown in Table 2. A total of 19 combinations with 9 antisera showed reasonable titers (++ and +++ in Table 2). Many researchers have used a solubilization system based on nonionic detergents to dissolve PCDDs and PCDFs in an aqueous medium.[14,18,19,29] Previous work by Sherry et al.,[28] and our current work, show that the use of DMSO in the ELISA leads to improved assay performance. We screened antibodies using 33% DMSO initially. In spite of this high level of DMSO, many antisera showed good recognition for these coating antigens. Haptens **IV**-BSA, **XI**-BSA, and **XII**-BSA showed low titers with DMSO but showed high titers without DMSO. Both **VII**-BSA and **XIII**-BSA demonstrated high titers regardless of DMSO concentration. Antibodies vary dramatically in their susceptibility to the denaturing effects of organic solvents. Our results indicate that some hapten structures led to antibodies with a high tolerance to organic solvents. Results in Table 2 also show the animal-to-animal variability in antibody titers and in binding to a given coating antigen. For example, rabbits 2114, 2525, and 2549 each bound differently to coating antigen **VII**-BSA. A rabbit that was a strong responder (i.e., 2549) generally showed a higher response to every coating antigen compared to the weaker responding 2114 and 2525.

**Screening for Inhibition.** All combinations of rabbit antiserum and coating antigen giving an absorbance response of >0.25 were screened for inhibition by 500 and 5000 pg/well TMDD (Table 3). Two concentrations of TMDD were tested to give an indication of the concentration dependence of the inhibition. Most antisera showed less than 50% inhibition (Table 3). Only antiserum 7598 with coating antigen **VII**-BSA showed more than 50% inhibition to TMDD. Based on this result, this assay was further developed. Homologous systems were not screened since experience has shown that more sensitive assays generally result from heterologous systems.[30]

**Solvent Effect**. To evaluate the tolerance of the assay to DMSO, various ratios of DMSO/PBSTB buffers (0−100% DMSO in PBSTB) were used to prepare the analyte solutions (Table 4). The concentration of DMSO given here is the value in the analyte solution. The final concentration of DMSO in the well was half of the value because the same amount of analyte solution and antiserum was added to the well (50 $\mu$L of analyte and 50 $\mu$L of antiserum).

An optimal DMSO concentration was chosen based on the $IC_{50}$ values and the ratios of the maximum and minimum absorbances for the sigmoidal standard curves ($A/D$). A low $IC_{50}$ value and a high value for $A/D$ are desirable. The $IC_{50}$ decreased as the DMSO concentration increased from 0 to 50% (0−25% in the assay wells), reaching a value of 21 pg/well. The trend to decreasing $IC_{50}$ in 0−50% DMSO may be due to the increased solubility of TMDD in the corresponding DMSO/PBSTB buffer. The $IC_{50}$ was essentially unchanged (16 pg/well) at 75% DMSO, and it increased sharply (181 pg/well) at 100% DMSO. The ratio $A/D$ increased as the DMSO concentration was increased from 0 to 50% to a value of 8.8. The value decreased to 7.1 and 2.0 at 75 and 100% DMSO, respectively. A DMSO concentration of 50% was selected for subsequent work based on a low $IC_{50}$ value and high $A/D$ ratio.


(26) Crosby, D. G.; Wong, A. S.; Plimmer, J. R.; Woolson, E. A. *Science* **1971**, *173*, 748−749.

(27) Qin, Z. *Chemosphere* **1996**, *33*, 91−97.

(28) Sherry, J. P.; ApSimon, J. W.; Collier, T. L.; Albro, P. W. *Chemosphere* **1990**, *20*, 1409−1416.

(29) Luster, M. I.; Albro, P. W.; Chae, K.; Lawson, L. D.; Corbelt, J. T.; McKinney, J. D. *Anal. Chem*. **1980**, *52*, 1497−1500.

(30) Goodrow, M. H.; Sanborn, J. R.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. In *Immunoanalysis of Agrochemicals: Emerging Technologies*; Nelson, J. O., Karu, A. E.; Wong, R. B., Eds.; ACS Symposium Series 586; ACS Publications: Washington, DC, 1995, pp 119−139.

Table 3. Percent Inhibition Using TMDD as an Inhibitor at Two Concentrations in Various Unoptimized ELISA Systems[a]

| | coating antigen | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IV-BSA | | VII-BSA | | X-BSA | | XI-BSA | | XII-BSA | | XIII-BSA | |
| rabbit no. | 500 | 5000[b] | 500 | 5000 | 500 | 5000 | 500 | 5000 | 500 | 5000 | 500 | 5000 |
| 69 | | | – | – | – | – | – | – | – | – | 18 | 23 |
| 89 | homol[c] | | 1 | 5 | 30 | 45 | *d* | *d* | 2 | 0 | 6 | 2 |
| 99 | | | 14 | 9 | 27 | 25 | *d* | *d* | *d* | 6 | *d* | *d* |
| 7598 | – | – | 61 | 68 | | | – | – | – | – | 3 | *d* |
| 7599 | – | – | 12 | 16 | homol | | 11 | 10 | *d* | *d* | 3 | 9 |
| 7600 | – | – | 29 | 36 | | | – | – | – | – | 14 | 18 |
| 5156 | – | – | – | – | – | – | | | – | – | – | – |
| 5163 | – | – | – | – | – | – | homol | | – | – | – | – |
| 5164 | – | – | 22 | 38 | – | – | | | – | – | 12 | 9 |
| 2114 | – | – | –7 | 3 | – | – | – | – | | | 5 | 5 |
| 2525 | – | – | – | – | – | – | – | – | homol | | – | – |
| 2549 | *d* | *d* | 4 | *d* | *d* | 0 | *d* | *d* | | | 6 | *d* |
| 2492 | *d* | *d* | 1 | *d* | 12 | 15 | 5 | *d* | *d* | 5 | | |
| 2493 | – | – | – | – | – | – | – | – | – | – | homol | |
| 2494 | 4 | 1 | 9 | 13 | 22 | 35 | 2 | 14 | 8 | 15 | | |

[a] The data shown are at a coating antigen concentration of 0.1 $\mu$g/mL and an antibody dilution of 1:10000. Other assay conditions as described in Materials and Methods. [b] With TMDD at 500 ang 5000 pg/well. [c] homol, homologous system in which the coating hapten is the same as was used for immunization. (–) absorbance <0.25 in Table 2 and were not tested for inhibition. [d] No inhibition.

Table 4. Effect of DMSO Concentration[a]

| DMSO (%) | $ABS_{max}$ (*A*) | slope (*B*) | $IC_{50}$ (pg/well) (*C*) | $ABS_{min}$ (*D*) | *A*/*D* | $R^2$ |
|---|---|---|---|---|---|---|
| 0 | 0.94 | 0.50 | 223 | 0.35 | 2.7 | 0.99 |
| 10 | 0.97 | 0.82 | 132 | 0.34 | 2.9 | 0.99 |
| 25 | 1.01 | 0.83 | 49 | 0.27 | 3.7 | 1.00 |
| 50 | 1.05 | 0.61 | 21 | 0.12 | 8.8 | 1.00 |
| 75 | 1.34 | 0.60 | 16 | 0.19 | 7.1 | 1.00 |
| 100 | 1.31 | 0.61 | 181 | 0.66 | 2.0 | 0.99 |

[a] ELISA conditions were 0.1 $\mu$g/mL coating antigen **VII**-BSA, 1:10000 dilution antiserum 7598. For preparation of assay conditions, PBSTB was employed instead of PBSB. Other incubation steps were held constant as described in the Materials and Methods section.

The results for inhibition studies with other solvents are presented in Table 5. The maximum absorbance (no analyte) ranged from 0.7 to 1.5 for all solvents tested with the exception of 2-methoxyethanol used above 10%. These values for 2-methoxyethanol (20−60%) ranged from 0.3 to 0.08, indicating that high concentrations of this solvent inhibited antibody−hapten binding. For methanol, DMF, acetonitrile, and ethyl acetate, assay inhibition by TMDD decreased with increasing organic solvent concentration. Inhibition increased with increasing concentration of ethanol, ethylene glycol, and *p*-dioxane. In all cases, inhibition by TMDD was less than that observed in 50% DMSO (75% inhibition), indicating that DMSO is the best cosolvent tested to date.

**Time Effect.** The effect of both first and second incubation times of the ELISA were studied. Five different incubation times were evaluated (Table 6). The 60-min first incubation resulted in an $IC_{50}$ of 27 pg/well, and a time of 90 min showed the lowest $IC_{50}$ (15 pg/well). The $IC_{50}$ values at 120, 180, and 240 min were 49, 53, and 81 pg/well, respectively. The *A*/*D* ratio was 4.7 at 60 min and then remained constant at 6.0 from 90 to 240 min. The 90-min second incubation time was selected for further work because of the low $IC_{50}$ and high *A*/*D* ratio at this time. Four different second incubation times (20−60 min) were evaluated, but no significant differences in the *A*/*D* ratio or $IC_{50}$'s were observed (data not shown).

**Effect of Detergent (Tween 20).** Figure 1 shows the results of experiments designed to test the effect of Tween 20 on the ELISA. Tween 20 is a nonionic detergent, and it has commonly been used in ELISA to reduce nonspecific interactions. In general, because dioxins are lipophilic, many researchers have used a solubilization system based on detergent micelles to dissolve PCDDs in an aqueous medium for their assays.[14,19,28] Tween 20 at a concentration of 0.05% in the assay buffer is used for most pesticide immunoassays. Thus, this concentration of Tween 20 was used for the initial screening. However, Vanderlaan et al.[18] reported that a high level (>0.5%) of Cutscum (technical-grade Triton X-100) in the final aqueous suspension made the ELISA for dioxin more sensitive. Chiu et al.[31] also reported that higher concentrations of Tween 20 improved the sensitivity for polychlorinated biphenyls (PCBs). Therefore, the effect of Tween 20 was tested. As shown in Figure 1, 0.05% Tween 20 influenced the ELISA strongly. The $IC_{50}$ in the standard curve with 0.05% (v/v) Tween 20 was 97 pg/well, whereas the $IC_{50}$ of the standard curve without Tween 20 was 9 pg/well. Manclús and Montoya[32] explained a similar result for 2,3,6-trichloropyridinol by reasoning that it might be related to nonspecific hydrophobic interactions between the detergent and nonpolar small organic molecules in an aqueous environment. The data in Figure 1 indicate that the use of Tween 20 increases the $IC_{50}$ of the ELISA. For this reason, Tween 20 was eliminated from the analyte solution and diluted antiserum in the ELISA.

**Reproducibility and Sensitivity.** Under optimized conditions [**VII**-BSA 0.1 $\mu$g/mL, antiserum 7598 1:10000 final dilution in the well, and TMDD in DMSO−PBSB buffer (1:1)], the average of 40 standard curves generated for a 1-month period showed the ELISA to be sensitive and reproducible. The average $IC_{50}$ was

(31) Chiu, Y.-W.; Carlson, R. E.; Marcus, K. L.; Karu, A. E. *Anal. Chem.* **1995**, *67*, 3829−3839.
(32) Manclús, J. J.; Montoya, A. *J. Agric. Food Chem.* **1996**, *44*, 3710−3716.

Table 5. Solvent Effect on the Percent Inhibition by the Surrogate Standard

| | % inhibition (50 pg/well[a] TMDD) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| concn (%) | methanol | ethanol | DMF | acetonitrile | ethyl acetate | 2-methoxyethanol | ethylene glycol | *p*-dioxane |
| 10 | 30[b] | 14 | 29 | 32 | 14 | 4 | 8 | 15 |
| 20 | 23 | 19 | 24 | 33[c] | 11 | *e* | 25 | 22[c] |
| 40 | 16 | 50 | 20 | 15 | 0[d] | *e* | 25 | 28 |
| 60 | 17 | 43 | 12 | 0 | −14%[c,d] | *e* | 30 | 31 |

[a] At 50% DMSO, this concentration of TMDD showed more than 75% inhibition. ELISA conditions were 0.1 μg/mL coating antigen **VII**-BSA, 1:10000 dilution antiserum 7598. For preparation of analyte solution and dilution of antiserum, PBSTB was employed instead of PBSB. [b] The percent inhibition is from a single experiment and calculated with the equation (1 − mean absorbance with inhibitor/mean absorbance without inhibitor) × 100. [c] Standard curves were only obtained with log−logit curve fit. [d] The organic solvent was not miscible with water and dissolved the polystylene plate. [e] No inhibition.

Table 6. Effect of the First Incubation Time[a]

| time (min) | $ABS_{max}$ (*A*) | slope (*B*) | $IC_{50}$ (pg/well) (*C*) | $ABS_{min}$ (*D*) | *A*/*D* | $R^2$ |
|---|---|---|---|---|---|---|
| 60 | 0.42 | 0.46 | 27 | 0.09 | 4.7 | 0.96 |
| 90 | 0.78 | 0.58 | 15 | 0.13 | 6.0 | 0.99 |
| 120 | 1.00 | 0.56 | 49 | 0.17 | 5.9 | 0.99 |
| 180 | 1.20 | 0.47 | 53 | 0.20 | 6.0 | 0.99 |
| 240 | 1.47 | 0.61 | 81 | 0.25 | 5.9 | 0.99 |

[a] ELISA conditions were 0.1 μg/mL coating antigen **VII**-BSA, 1:10000 dilution antiserum 7598. For preparation of assay conditions, PBSTB was employed instead of PBSB. Other incubation steps were held constant as described in the Materials and Methods section.

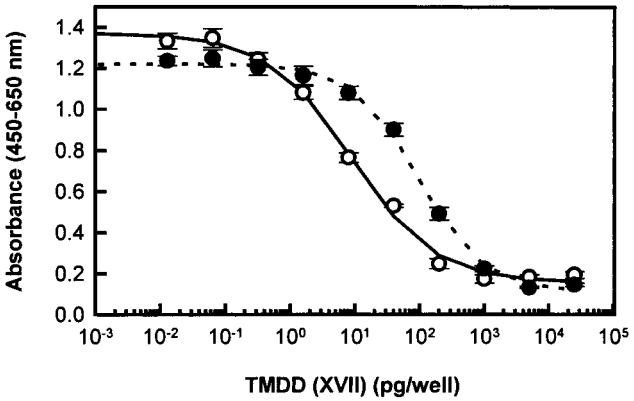



Figure 1. Effect of detergent (Tween 20): (○) 0%; (●) 0.05%. The parameters for the standard curve with Tween 20 are $ABS_{max}$ = 1.22, slope = 0.9, $IC_{50}$ = 97 pg/well, and $ABS_{min}$ = 0.11. The parameters for the standard curve without Tween 20 are $ABS_{max}$ = 1.37, slope = 0.7, $IC_{50}$ = 9 pg/well, and $ABS_{min}$ = 0.16.

12 ± 4 pg/well. The average CV of the individual points on the standard curve was 16.0% (Figure 2). For the representative standard curve, the means and CVs (in parentheses) for the parameters [the maximum absorbance ($ABS_{450}$ − $ABS_{650}$), slope, and minimum absorbance] were 1.1 (10%), 0.70 (12%), and 0.13 (16%), respectively. It is important to point out that a separate standard curve is generated for each plate. The precision and accuracy for three individual samples (2, 10, and 125 pg/well TMDD) were tested with 10 assay plates (Table 7). The average CVs for TMDD samples of 2, 10, and 125 pg/well were 37, 20, and 14%, respectively. The precision of the spiked samples was improved as the concentration increased. The average recovery rates were 50 (2 pg/well), 109 (10 pg/well), and 154% (125 pg/well). As expected, recoveries were most accurate (near 100%) for samples nearest the $IC_{50}$. The 2 and 125 pg/well samples

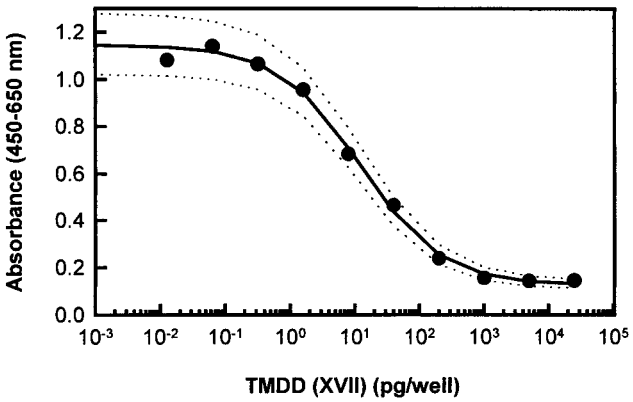



Figure 2. Standard curve for the ELISA, using coating antigen **VII**-BSA (0.1 μg/mL) and antiserum 7598 (diluted 1:10 000, final dilution in the well). The standard curve represents the average of 40 plates. The dotted lines are the average ± standard deviation.

Table 7. Spike Recoveries of Individual TMDD Samples[a]

| spike concn (pg/well) | av rec (pg/well) ($n$ = 10)[b] | av % CV ($n$ = 10) | av % rec ($n$ = 10) |
|---|---|---|---|
| 2 | 1.0 ± 0.4 | 37 | 50 |
| 10 | 10.8 ± 2.2 | 20 | 109 |
| 125 | 192 ± 28 | 14 | 154 |

[a] The TMDD samples were prepared individually with PBSTB:DMSO (1:1). [b] $n$, the number of the plates. Each value represents the average of four samples analyzed in quadruplicate on each plate.

inhibited the assay 20 and 85%, respectively. These inhibition levels are at the edge of the portion of the standard curve used for quantitation, and therefore, these measurements were less accurate.

The typical standard curve, obtained from quadruplicate wells at each concentration, indicated a limit of detection (LOD) of 0.5 pg/well for TMDD. The definition of LOD used here is the concentration of the absorbance value equal to the absorbance at zero concentration minus 3 times the standard deviation of the absorbance at zero concentration. The quantitative working range was established between the concentrations producing 20 and 90% inhibition, i.e., 2−240 pg/well.

In previous assays for TCDD, Stanker et al. reported 500 pg/well 2,3,7,8-TCDD as the LOD and 1000 pg/well as the $IC_{50}$ using monoclonal antibodies. Watkins' ELISA using the same antibodies showed an LOD of 100 pg/well and an $IC_{50}$ of 200 pg/well.[16,17,19] Langley's assay using polyclonal antibodies exhibited an $IC_{50}$ of

1000 pg/well. More recently, rapid tube test and microtiter plate tests were reported by Harrison and Carlson[21] using the monoclonal antibodies developed by Stanker et al. The tube test and microtiter plate test exhibited LODs of 100 pg/tube and 25 pg/well, respectively.

A number of aspects of the hapten design for these antibodies may have led to the improvements realized in detection limits and selectivity for the ELISA. Alkyl chains containing at least one double bond or aromatic ring in the chain were used for spacers. It was hypothesized that these spacers had enough rigidity to project and keep the hapten moieties away from the protein surface, aiding in the recognition of TCDD-haptens during antibody formation. For water-insoluble haptens, the role of the spacer may be much more important because the hapten has the ability to fold back on the protein surface or within the protein core after conjugation. A recent example from the literature described the development of an ELISA for PCBs used a flexible hexanoic acid spacer for generation of monoclonal antibodies, and only modest success was achieved with respect to assay sensitivity.[31,33] Second, the 2-position substitution was used to attach the spacers, instead of the 1-position substitution previously reported, and this pattern more closely mimics the substitution pattern of 2,3,7,8-TCDD during antibody formation.[14,16−19] Third, spacers containing only hydrocarbons reduced the handle recognition. Cross-reactivity data for monoclonal antibodies generated using previously reported haptens indicated that the polar carboxamide moiety used to link the spacer to the dioxin ring is strongly recognized by these antibodies.[17] Fourth, the heterologous ELISA format used in this work may also contribute to the increased sensitivity,[30] compared to previously used homologous formats.[14,16−20] Finally, the use of high concentrations of DMSO in the assay buffer and the removal of detergents from the assay improved the performance.

**Comparison of the Standard Curves for TCDD and the Surrogate Standard TMDD.** One of our overall goals for this project is the development of a surrogate standard. That is a compound that will behave like 2,3,7,8-TCDD under analytical conditions but is less toxic. Such a compound would allow laboratories that are restricted in their ability to deal with TCDD waste to conduct these assays and would provide a useful internal standard for other analytical methods. The standard curve for TMDD showed it is a useful surrogate standard for detection of 2,3,7,8-TCDD (Figure 3). Two sets of serially diluted TMDD and 2,3,7,8-TCDD solutions were analyzed on each plate. These two standard curves were obtained from six plates on three different days. Although, these two standard curves appeared to be the same visually, rigorous tests of similarity were conducted using nonlinear approaches.[34] De Lean et al.[35] have developed an excellent program for this purpose (ALLFIT). This program offers simultaneous fitting of sigmoidal curves using the four parameter logistic equation by an *F* test. When the two curves are forced to have the same value for all four parameters, the program calculates the loss in fit, compared with the first fit that had no constraint, by an *F* test. From the resulting *p* values we can decide

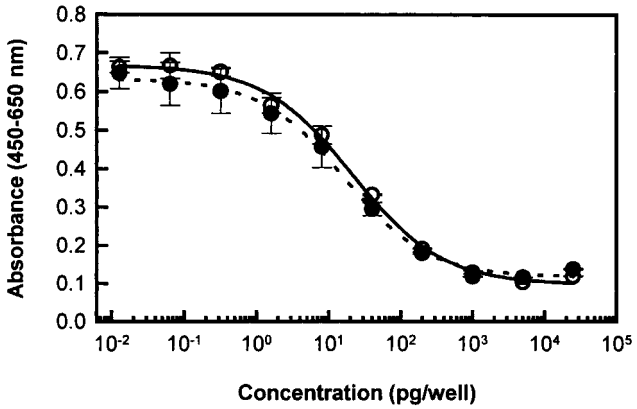



Figure 3. Standard curves for 2,3,7,8-TCDD (●) and the surrogate standard (TMDD) (○). Each point represents the average ± standard deviation for quadruplicate measurements.

Table 8. Characteristics of Standard Curves Obtained for Different Surrogate Standards

| | $IC_{50}$ (pg/well) (*C*) | LOD (pg/well) | slope (*B*) |
|---|---|---|---|
| DMDD (**XVIII**) | 103 | 19 | 0.64 |
| TMDD (**XVII**) | 12 | 0.2 | 0.66 |
| 2,3,7,8-TCDD | 14 | 0.8 | 0.76 |

whether or not the experimental points deviate significantly from the theoretical curves. If the *p* value is <0.05, the deviation of the combination curve, calculated from the two curves, is highly significant from each individual curve. If $p > 0.05$, the individual curves are not significantly different. In this case, the *F* test had a *p* value of >0.05 ($p = 0.462$, DF = 18, $F = 0.001\,76$). Thus these two curves were statistically similar. In marked contrast to TMDD, the *F* test for 2,3−dichloro-7-methyl-dibenzo-*p*-dioxin (DMDD; **XVIII**) (Table 8) showed a *p* value much lower than 0.05 ($p = <0.000001$, DF = 18, $F = 47.3$). That is, the standard curves for 2,3,7,8-TCDD and DMDD were significantly different. As shown in Table 8, DMDD had a very different $IC_{50}$ relative to TMDD and TCDD. Thus, the *F* test should indicate that these two curves were significantly different and this was the case.

The ELISA response to DMDD was compared to the response with TMDD and 2,3,7,8-TCDD (Table 8). In spite of the similarity in slopes, the affinity of DMDD was lower than that of TMDD (the $IC_{50}$ values were 10-fold higher than those of TMDD). This indicated that the TCDD-like substitution pattern was critical for the surrogate standards to bind as well as TCDD. Because of the decreased toxicity of TMDD[24] and its identical response to that of TCDD in the ELISA, we found TMDD to be useful as a surrogate standard for 2,3,7,8-TCDD.

**Cross-Reactivities.** The cross-reactivity of the ELISA was evaluated by using various dioxin congeners, dibenzofurans, and polychlorinated biphenyls. The results of the cross-reactivity study are presented in Table 9, and the general structures of PCDDs, PCDFs, and PCBs are in Figure 4. For determination of cross-reactivity, the $IC_{50}$ of TMDD was assigned a value of 100%, and the cross-reactivities for other compounds are reported according to their $IC_{50}$'s relative to this value. The $IC_{50}$ of TMDD was 12 ± 4 pg/well. The ELISA recognized 2,3,7,8-TCDD virtually identically to TMDD. The data suggest some general points concerning the cross-reactivity of the ELISA. (1) The cross-reactivity of the

(33) Johnson, J. C.; Van Emon, J. M. *Anal. Chem*. **1996**, *68*, 162−169.
(34) Grotjan, H. E.; Keel, B. A. In *Immunoassay*; Diamandis, E. P., Christopoulos, T. K., Eds.; Academic Press: San Diego, 1996; pp 51−93.
(35) De Lean, A.; Munson, P. J.; Rodbard, D. *Am. J. Physiol.* **1978**, *235*, E97-E102.

Table 9. Cross-Reactivity for the ELISA[a] for TCDD

| competitors | cross-reactivity (%) |
|---|---|
| surrogate standard | |
| TMDD | 100 |
| dibenzodioxins | |
| 1-CDD | <0.1 |
| 2,7-DiCDD | 0.3 |
| 2,3,7-TriCDD | 31 |
| 1,2,3,4-TCDD | <0.1 |
| 1,3,7,8-TCDD | 68 |
| 2,3,7,8-TCDD | 109 |
| 1,2,3,7,8-PentaCDD | 110 |
| 1,2,3,4,7,8-HexaCDD | 1 |
| OCDD | <0.1 |
| dibenzofurans | |
| 2,3,7,8-TCDF | 77 |
| 2,3,4,7,8-PentaCDF | 3 |
| PCB | |
| 3,3′,4,4′-TCB | 0.1 |

[a] ELISA conditions were 0.1 $\mu$g/mL coating antigen **VII**-BSA, 1:10000 dilution antiserum 7598. Preparation of assay conditions were as described in the Materials and Methods section. Cross-reactivity is determined by expressing the ratio of the $IC_{50}$ of the chemical assigned to be 100% (TMDD) to the $IC_{50}$ of the other compounds and expressed as percent.

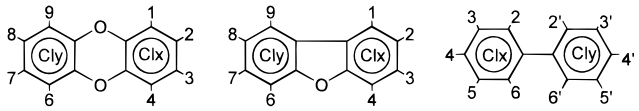



Figure 4. Structures of PCDDs, PCDFs, and PCBs.

ELISA for octachloro-dibenzo-*p*-dioxin is very low (<0.1%). Octachloro-dibenzo-*p*-dioxin is the predominant PCDD in many real samples such as sediments,[2] and therefore, it is important that ELISAs for TCDD do not show significant cross-reactivity for this compound. (2) Compounds tested with three, four, or five chlorine atoms in a substitution pattern similar to that of 2,3,7,8-TCDD exhibited relatively high cross-reactivities such as 1,2,3,7,8-TriCCD (110%). Exceptions are 2,3,4,7,8-PentaCDF (3%) and 3,3′,4,4′-TCB (0.1%). It is not obvious why the cross-reactivity of 2,3,4,7,8-PentaCDF is so low, especially when one considers the cross-reactivity of 1,2,3,7,8-PentaCDD and 2,3,7,8-TCDF (77%). The polychlorinated biphenyl may simply be too structurally dissimilar to TCDD to cross-react strongly. However, significantly more extensive cross-reactivity data would have to be generated to clearly understand the low cross-reactivity of these two structures. (3) Structures with a 1,2,3,4 substitution pattern showed very low cross-reactivities. (4) Reduction from a congener with three chlorine atoms (2,3,7-TriCDD) to one with two (2,7-DiCDD) results in a dramatic drop in cross-reactivity (from 31 to 0.3%).

The ELISA developed in this work is significantly more selective than either the RIA reported by Albro and co-workers[14] or the ELISA developed by Stanker and colleagues.[17] Our ELISA and Albro's RIA showed similar selectivity relative to TCDD for 1,2,3,7,8-PentaCDD and 2,3,7,8-TCDF; however, the ELISA is dramatically less selective for 2,7-BiCDD and OCDD (100- and 80-fold, respectively). Of the compounds tested using both our ELISA and the Stanker and co-workers assay, the Stanker ELISA only exhibited comparable selectivity for 1,2,3,7,8-PentaCDD. Cross-reactivities of our ELISA were lower for 2,7-DiTCDD (1000-fold), 1,3,7,8-TCDD (10-fold), 1,2,3,4,7,8-HexaCDD (10-fold), and 2,3,7,8-TCDF (3-fold).

## CONCLUSIONS

Careful development and optimization of an ELISA to TCDD using new polyclonal antibodies has resulted in an immunoassay with an $IC_{50}$ at least 6-fold lower than previously reported RIAs and ELISAs.[14−21] This new ELISA can detect as little as 0.5 pg/well 2,3,7,8-TCDD and has an $IC_{50}$ of only 12 pg/well for this compound. In addition, this new assay is more selective for this most toxic dioxin congener compared to previous immunoassays. This work shows that, through careful hapten design and assay development, sensitive and selective immunoassays can be developed for highly lipophilic compounds using polyclonal antibodies.

This work also demonstrates the practical use of a surrogate standard for analysis of dioxins and other toxic compounds. The surrogate used here (TMDD) is an effective but far from ideal surrogate for TCDD. This compound behaves almost identically to TCDD in the ELISA and is less toxic. However, improved surrogates could be developed. For example, a surrogate that could also be easily isotopically labeled is desirable. Also, there is much room for further reduction of the toxicity of the surrogate. These goals are the object of ongoing work.

The development of this new ELISA also will require the development of sample preparation methods that are compatible with the assay and validation of the assay with real samples contaminated with PCDDs. Traditional sample preparation methods for TCDD work well with GC/MS techniques but are often not compatible with ELISAs. Sample preparation methods such as microwave extraction with more polar solvents or supercritical fluid extraction could prove to be useful. Successful development of immunoassay-compatible sample preparation methods for PCDDs will make the ELISA a practical tool for these analyses.

## ACKNOWLEDGMENT

This project was funded by the NIEHS Superfund Basic Research Program ES04699, USEPA Center CR819658, NIEHS Center ES05707, and UC Presidents Campus Laboratory Collaboration 95-103. S.D.G. was funded by an NIEHS Postdoctoral Fellowship ES05697-02. We thank Cosmo Oil Co., Ltd. for their support of this project. B.D.H. is a McMasters' Fellow of the CSIRO (Canberra, Australia) and an NSF Fellow, INT-96-02893. Although portions of this work were supported by the U.S. Environmental Protection Agency, no endorsement of the information contained herein is implied.

Received for review July 29, 1997. Accepted October 7, 1997.[⊗]

AC9708203

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1997.