A HIGHLY SENSITIVE DIOXIN IMMUNOASSAY AND ITS APPLICATION TO SOIL AND BIOTA SAMPLES

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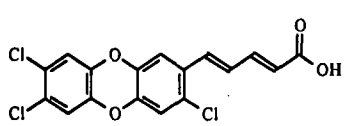
Introduction

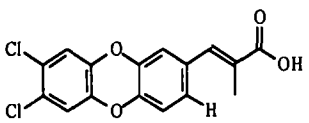
PHDDs are a well-known group of highly toxic chemicals present in nearly all components of the global ecosystem, including air, soil, sediment, fish and humans [1, 2]. Dioxin analysis is equipment intensive and expensive requiring low ppt or even ppq levels of detection. A simple, rapid, cost effective method of analysis is desired to enable researchers to explore issues involving dioxin more quickly and to make more rational regulatory decisions. Immunoassay (IA) may be an ideal screening and semi-quantitative technique to fill such a need. However, due to the lipophilic properties and the difficulty of the chemistry of PCDDs, only a few attempts to detect TCDD by IA have been reported [3-5], and the sensitivity and tolerance to sample matrices for these assays are still far below satisfaction. Based on careful hapten design and synthesis, a sensitive polyclonal antibody-based ELISA was developed in this laboratory [6]. In this study, this assay was further screened and optimized with new coating antigens, and validated by GC-MS with biota samples. A simple cleanup method was also developed for soil sample monitoring.

Methods and Materials

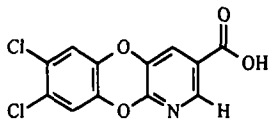
Haptens used in this study are listed as follow:



Immunogen hapten



Hapten I



Hapten II

ELISA format was similar to that previously described by Shan et al. [7]. Assay was optimized by testing two-dimensional titration and solvent effect. Cross-reactivity were tested for compounds listed in Table 1 by preparing each compound in 50% DMSO-PBS.

Validation. Extracts of fish, egg samples and human milk were tested by optimized immunoassay. The results were compared to GC/MS data provided by the USGS and Cosmo Research Institute.

Soil Matrix Effects and Sample Cleanup. Soil sample was placed in a glass flask, and spiked with ^{14}C-TCDD. Samples were extracted with 15mL of solvent. After separation of solvent, another 15 mL of solvent was added for second extraction. The combined solvent was then evaporated to a volume of 1 mL. The sample aliquots were directly measured by LSC and immunoassay, or column cleanup [8].

Results and Discussion

New Antigen Hapten Design and Synthesis. To develop a sensitive and specific assay, rational design of a coating antigen hapten is very important. In the competitive ELISA format, assay

sensitivity is determined by the difference in affinity between two competitive components (coating antigen and analyte of interest) with antibody. Theoretically, an IA sensitivity is determined by antibody affinity. The ultimate detection limit of an assay is approximately 10-100 times lower than the K_d of antibody. The K_d of Ab 7598 used in this study was measured by accelerator mass spectrometry [9] and found to be 1.0×10^{-10} M. The I_{50} of a sensitive IA based upon this antibody by Sugawara et al. [6] was 0.75 nM (lower detection limit LDL 0.1 nM), which is far below the theoretical capability of this antibody. Thus, one of our objectives was to improve the assay sensitivity by the design of the antigen hapten. The best antigen hapten selected in the previous report [6] was hapten I, which has similar structure to immunogen hapten except for the replacement of Cl with H at position 8. We designed and synthesized hapten II as a new coating antigen hapten, in which the second benzene ring was replaced with a pyridine ring. Due to the contribution of the extra electron cloud of N in the ring, it is more structurally different from the immunogen hapten and target analyte TCDD, and thus has lower affinity to Ab7598.

Assay Optimization. The optimal assay conditions for the new system was obtained from antibody titration and solvent effect studies. The best concentration for II-BSA with Ab7598 was 0.5 μg/mL, and the final concentration for Ab7598 used in subsequent study is 1:7,000 dilution. Dioxin is highly lipophilic and will adhere to glass, plastic or other particle surfaces, higher concentration of co-solvent is very important for consistent assay performance and sensitivity [7]. In this system, a DMSO concentration of 50% was selected for subsequent experiments. The I_{50} of this assay was 36 ng/L with a LDL of 4.0 ng/L (0.012 nM. In the previous system, Sugawara et al. [6] reported 240 ng/L TMDD as the I_{50} and 40 ng/L as LDL with same antibody. Approximately ten times better sensitivity was achieved by this new system. The ultimate sensitivity of a competitive ELISA is limited by the antibody affinity constant, the random experimental error, and precision of detection system [10]. The lowest detection limit possible for a competitive immunoassay would be 10^{-10} M with a $K_d = 10^{-8}$ M, a 1% coefficient of variation for the response at zero dose. In this new system, the assay detection limit of TMDD is 1.2×10^{-11} M, which is about 10 times lower than the antibody K_d (1.0×10^{-10} M). Therefore, rational hapten design can be a useful approach to obtain a sensitive assay near the theoretical limit.

Assay Validation. Extracts from fish, egg and human milk samples were analyzed by both GC-MS and ELISA in a blind fashion (Figure 1). A good agreement between GC-MS and ELISA measured TMDD equivalent was obtained from linear regression analysis ($y = 0.97x + 0.05$, $R^2 = 0.92$). A fairly good correlation between ELISA and TEF values was also observed with these samples (Figure 2). The slope value of the linear regression equation is less then 1, which means an overestimation by ELISA in comparison to TEF values. However, a strong correlation ($R^2 = 0.84$) between ELISA and TEF suggests that this ELISA is useful for TEF screening of dioxins in these samples.

Soil Matrix Effects and Sample Cleanup. To find a good solvent for efficient extraction, seven different solvents or combinations were screened in spiked soil samples. Three solvents (hexane, CH_2Cl_2, and MeOH/CH_2Cl_2) can efficiently extract TCDD from soil. These solvents have an extraction recovery > 86%. With a Florisil column cleanup step, the total recoveries of ^{14}C-TCDD for these three solvents were from 78.3 to 80.3%. Other solvent (MeOH, DMSO, hexane/MeOH and CH_2Cl_2/MeOH (1:1)) gave poor recoveries from extraction. The soil matrix effects differed with the extraction solvents used. The hexane method gave the least interference for the ELISA

and a 128 times dilution eliminated the matrix effects. Furthermore, about 4-8 fold of this interference can be effectively removed by Florisil column cleanup. The extracts from the $MeOH/CH_2Cl_2$ method had the strongest matrix effects in the immunoassay. Similar to hexane, the CH_2Cl_2 method had good extraction recovery and slightly higher matrix effects than hexane. Due to public concerns about the potential problem with chlorinated solvents, hexane was chosen in this study for soil sample preparation for dioxin immunoassay. Three field samples were tested for method validation. Either with or without column cleanup, ELISA data showed good agreement with GC/MS results for all three samples. These results suggest that this ELISA can be used as a rapid screen and predict dioxins in the soil samples without additional cleanup steps.

In conclusion, rational hapten design and preparation, and further optimization of an ELISA resulted in a highly sensitive dioxin IA which could detect low ppt levels of dioxin. A high concentration of co-solvent (DMSO) in this ELISA system is extremely important for the accurate performance of the ELISA for the highly lipophilic dioxins. A good correlation between this ELISA and TEF values for biota sample extracts indicates that this assay can be used as a TEF screening method for dioxins and PCDFs on its own or sequentially to a more general screen dased on the Ah receptor [11]. Finally, a simple and rapid sample preparation method was developed with reasonable recovery. By combining this extraction method with the ELISA reported here, one can carry out effective dioxin screens in a fields setting.

Acknowledgements

We wish to thank Drs. D.E. Tillitt and J. Zajicek, USGS, Columbia, MO for biota sample extracts and the GC/MS data, and Mr. Brad Call, Environmental Engineering Branch, US Army Engineer District, Sacramento, CA for providing field soil samples and corresponding GC/MS data. This project was funded by NIEHS Superfund Research program, 5 P42 ES04699-08, the NIEHS- CEHS and EPA-CEHR, CR819658.

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Table 1. Comparison of toxic equivalency (TEF) value and immunoassay cross-reactivity for CDDs, PCBs and CDFs compounds

	Congener	Cross-reactivity %	TEF Value
Surrogate standard	TMDD	100	--
PCDDs	1-CDD	<0.01	<0.001
	2,7-DiCDD	0.19	<0.001
	2,3,7-TriDD	6.7	<0.001
	1,3,7,8-TCDD	43	0.1
	1,2,3,4,-TCDD	0.01	<0.001
	2,3,7,8-TCDD	129	1.0
	1,2,3,7,8-PentaCDD	72.9	1.0
	1,2,3,4,7,8-HexaCDD	1	0.1
	1,2,3,4,6,7,8-HeptaCDD	0.3	0.01
	OCDD	<0.01	0.001
	2-Br,3,7,8-TriCDD	110	1.0
	2,3-DiBr,7,8-DiCDD	115	1.0
PCBs	3,3',4,4'-TCB	0.10	0.0001
	3,3',4,4',5-PCB	<0.01	0.1
	3,3',4,4',5,5'-HCB	<0.01	0.01
PCDFs	2,3,7,8-TCDF	26	0.1
	2,3,4,7,8-PentaCDF	9.0	0.5
	1,2,3,7,8-PentCDF	0.1	0.05
	1,2,3,6,7,8/1,2,3,7,8,9-HCDF	5.4	0.1
	1,2,3,4,7,8-HCDF	<0.01	0.1
	1,2,3,4,6,7,8-HeptaCDF	0.06	0.01
	OCDF	<0.01	0.0001

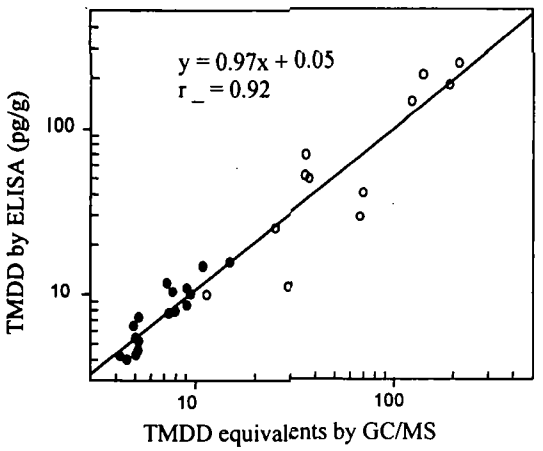

Figure 1. Relationship between dioxins (TMDD equivalent) measured by GS-MS and ELISA. (○) represents fish and egg sample extracts (USGS, Columbia, MO), and (•) represents milk extracts (Cosmo Research Institute, Japan). $y = 0.97x + 0.05$, $R^2 = 0.92$, $n = 29$.

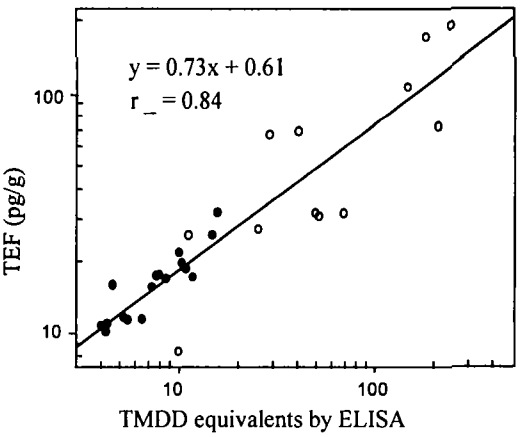

Figure 2. Relationship between TMDD equivalents by ELISA and TEF values calculated upon GCMS and IA cross reactivity results. $Y = 0.73x + 0.61$, $R^2 = 0.84$. (○) represents fish and egg samples from USGS, and (•) represents milk extracts from Cosmo Research Institute, Japan.