A New Dioxin/Furan Immunoassay with Low Picogram Sensitivity and Specificity Appropriate for TEQ Measurement

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INTRODUCTION

Regulation of PolyChlorinated DibenzoDioxins and PolyChlorinated DibenzoFurans (PCDD/Fs) is moving toward the use of Toxic EQuivalence (TEQ), which estimates the total toxicity of a sample. Accordingly, PCDD/F analysis generally focuses on the 17 congeners which have the highest Toxic Equivalency Factors (TEFs), all of which contain the 2,3,7,8- chlorination pattern. Many researchers and regulators see great value in an analytical tool capable of rapid and direct measurement of sample TEQ. More than two decades of research[1-3] have been directed toward developing rapid PCDD/F measurement capability by immunoassay. However, previous dioxin immunoassays have failed primarily because of inadequate sensitivity. A new Enzyme ImmunoAssay (EIA) for PCDD/Fs has been developed which has exceptional sensitivity for 2,3,7,8-TCDD and a dioxin/furan cross-reaction profile which is suitable for TEQ measurements. This EIA is designed to measure TEQ by responding to the toxic PCDD/F congeners in approximate correlation with their TEFs. Studies to compare the EIA to HRGC/HRMS analysis are in progress in several laboratories using several sample matrices. These studies will focus first on conventional sample preparation protocols to validate the EIA independent of sample preparation. These protocols will then be modified to reduce sample cleanup to the minimum tolerated by the EIA. Later studies will initiate development of EIA specific sample preparation protocols to maximize the economic benefit of the test.

MATERIALS AND METHODS

EIA Development. The rabbit polyclonal anti-dioxin antibodies and competitor-enzyme conjugates used in this EIA will be described in a subsequent publication[4].

Sample Preparation and Solvent Exchange. PCDD/F samples are typically prepared using organic solvents which are incompatible with the EIA. Standards or extracts of samples which have been prepared by conventional methods can be dried and redissolved in methanol for EIA analysis. During this solvent exchange, the PCDD/Fs are retained within a thin layer of detergent. Methanol is added to redissolve the PCDD/Fs and this solution (10 μL) is added directly to the EIA tubes. It should be noted that the solubility of 2,3,7,8-TCDD in methanol is 10 ppm[5], far above the levels typically encountered in this procedure.

EIA Procedure. A previously described protocol[2,3], based on antibodies immobilized on plastic tubes, has been modified for this EIA. An initial sample incubation allows binding of PCDD/Fs from solution by the immobilized anti-dioxin antibodies. After washing of the tubes, a competitor-horseradish peroxidase (HRP) conjugate is added to the tubes, allowing the conjugate to bind to antibodies not occupied by PCDD/Fs. After washing of the tubes to remove unbound conjugate, a chromogenic HRP substrate is added to allow color development by the bound enzyme. The resulting optical density is directly proportional to the amount of enzyme bound and is inversely related to the PCDD/F concentration in the original sample. PCDD/F concentrations in unknown samples are inferred from a standard curve. Results determined from the standard curve must be related to the original sample concentration by back calculation using the proper dilution and volume factors. Total EIA time from a prepared sample is about 3 hours.

RESULTS AND DISCUSSION

Sensitivity and Reproducibility. Figure 1 shows combined results for 18 standard curves of 2,3,7,8-TCDD run over a 5 week period. Response values are expressed as a percentage of the negative control, which is methanol plus 0.2 ppm Triton X-100. The detection limit is approximated by the I_{85}, the concentration giving 85% of the negative control OD. The observed detection limit was 3.8±0.6 pg/tube (mean±SD). The midpoint of the curve, defined as the I_{50} or the concentration giving 50% of the negative control OD, was 19.5±2.2 pg/tube. This sensitivity is sufficient to detect 2,3,7,8-TCDD in a 10 μL aliquot at concentrations lower than the lowest calibrator specified for US-EPA methods such as 8290 and 1613[6]. Such sensitivity is competitive with HRGC/HRMS methods. The EIA requires a small enough volume that prepared extracts could be split for EIA analysis without consuming an excessive fraction of the whole. For example, the EIA could detect 10 pg/g of 2,3,7,8-TCDD in a soil, using less than 10% of the extract from a 10 gram sample.

Specificity. The anti-dioxin antibody used in this EIA binds to PCDD/F congeners with different affinities. The specificity of the test is predominantly for PCDD/Fs which contain 3 to 6 chlorines and the 2,3,7,8 substitution pattern. Crossreactivity data relative to 2,3,7,8-TCDD are given in Table 1. Test specificity roughly parallels the TEF values of the individual PCDD/F congeners. As a result, the test should be useful for TEQ measurement. Sensitivity for PCBs is negligible, except for slight recognition of PCBs 77 and 126, the congeners which are most similar in structure to 2,3,7,8-TCDD.

Detergent Tolerance of the EIA. The effect of Triton X-100 on EIA sensitivity was tested because of past use of this detergent for sample solubilization during immunoassay analysis of dioxin[7]. Methanol solutions of Triton X-100 were added to the EIA tubes prior to the addition of standards in methanol. Table 2 shows that the effect on the sensitivity to 2,3,7,8-TCDD was minimal if the Triton level was significantly below Triton's critical micelle concentration (CMC) of 150 ppm[8]. The I_{50} increased slightly near the Triton CMC and was still within twofold of the control above the CMC. These results indicate that the high load of Triton X-100 which might be used for solubilizing samples[7] will still allow analysis by this EIA. This detergent tolerance should facilitate coupling the EIA to newer sample preparation methods such as cloud point extraction[9].

Detection of 2,3,7,8-TCDD Spiked into Soil Extracts. Acetone:hexane (1:1) extracts of 3 soils were washed with water and the hexane supernatant was cleaned with concentrated H_2SO_4. These extracts were spiked with 2,3,7,8-TCDD and analyzed by EIA (Table 3). The percent recovery values for the three soils demonstrate that the EIA is capable of detecting 2,3,7,8-TCDD at low picogram levels in the presence of a large amount of crude soil matrix. The conditions of this experiment mimic direct soil analysis at 1 ppb by using a 10 pg 2,3,7,8-TCDD spike in extract from 10 mg of soil (10 pg/10 mg = 1 ng/g).

Validation of TEQ Measurement Concept. The EIA response of a sample can be predicted using a simple additive response model[3] which combines immunoassay cross-reactivity, congener concentrations determined by GC/MS, and TEFs. This model was applied to a set of 20 fish samples previously analyzed by HRGC/HRMS. The results (Figure 2) demonstrate excellent correlation ($r = 0.987$) between predicted EIA response and measured TEQ. These data indicate that the specificity of this EIA will allow measurement of sample TEQ if the sample can be properly prepared and introduced into the EIA. This validation of the concept of TEQ measurement justifies the additional effort required for further method development work.

Alternative Internal Standard Method and Screening Strategy. Immunoassays can not discriminate based on mass and would therefore be unable to distinguish between native analyte and mass-labeled standards. This EIA shows both high sensitivity and broad specificity within the toxic PCDD/Fs. These properties will make it difficult to use this EIA for analysis of samples which contain conventional internal standard mixtures. For example, a sample with background levels of native PCDD/Fs plus a spike of 100 pg ^{13}C-2,3,7,8-TCDD in a 10 μL aliquot would give a nearly full scale EIA response. The high background signal due to such internal standards would lead to an unacceptable loss of EIA sensitivity to low levels of native material. Because of this conflict, we are

developing an internal standard protocol using mass-labeled PCDD/F congeners which are not reactive in the EIA. Such a protocol would be compatible with both EIA and HRGC/HRMS, removing the need to prepare a separate extract for each analytical method. A sample which was spiked and prepared conventionally for HRGC/HRMS analysis could be subsampled at any point in the preparation process for EIA analysis, according to the schematic diagram in Figure 3. In this screening strategy, most negative samples would not require further analysis, thereby avoiding a portion of the cost for complete HRGC/HRMS analysis. Positive samples could be confirmed by completion of the conventional method using the remainder of the original extract. Maximum economic benefit in the strategy of Figure 3 would be realized by removing a subsample for EIA analysis as early as possible in the sample clean-up process. A similar strategy[1] has been used successfully for several years in the immunoassay analysis of samples for PCBs, petroleum fuels, PAHs, and several other analytes[10,11]. The use of this field screening strategy has dramatically reduced cost per sample while decreasing the analytical cycle time from several days to a fraction of a day.

ALPHA KIT EVALUATION PROGRAM

The reagents described here have been used to prepare kits for evaluation by five independent laboratories in Canada, Germany, and the United States. This evaluation program is designed to yield information which will guide the development of protocols for preparation of several sample types, including soils, sediments, fly ash, fish tissue, and stack gases. Initial studies will emphasize the use of conventionally prepared extracts and will rely on negative samples for evaluation of matrix interferences. Matrix tolerance data developed in this phase will determine the degree of extract cleanup required for the EIA to reach various sensitivity targets. Contaminated samples prepared by these partial cleanup protocols will be analyzed by EIA and correlated to HRGC/HRMS data to validate the protocols. Evaluation of immunoassay specific sample preparation methods will begin as soon as progress permits. Because stack gas sampling protocols do not allow storage of unspiked samples for later analysis, EIA work on this matrix will be based on the use of the alternative internal standard procedure described above.

ACKNOWLEDGMENT

Thanks to Eric Reiner for the fish data used in Figure 2.

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Table 1. Specificity of the EIA. Response curves were determined for each congener. The percent crossreactivity = (((congener I_{50}) ÷ (2,3,7,8-TCDD I_{50})) x 100). Measured values are typically based on 2 to 4 independent curves, each containing at least 4 concentrations. Percent crossreactivity values in parentheses are estimates based on related congeners. These results show that the EIA response to PCDD/F congeners correlates approximately to TEF.

Compound and TEF		Percent Crossreactivity	Compound and TEF		Percent Crossreactivity
Toxic Dioxin Congeners	TEF		Toxic Furan Congeners	TEF	
2,3,7,8-TCDD	1	100	2,3,7,8-TCDF	0.1	18
1,2,3,7,8-PeCDD	0.5	54	1,2,3,7,8-PeCDF	0.05	(10)
1,2,3,4,7,8-HxCDD	0.1	<0.1	2,3,4,7,8-PeCDF	0.5	31
1,2,3,6,7,8-HxCDD	0.1	12	1,2,3,4,7,8-HxCDF	0.1	(<0.1)
1,2,3,7,8,9-HxCDD	0.1	(6)	1,2,3,6,7,8-HxCDF	0.1	(3)
1,2,3,4,6,7,8-HpCDD	0.01	(<0.1)	1,2,3,7,8,9-HxCDF	0.1	(3)
OCDD	0.001	(<0.1)	2,3,4,6,7,8-HxCDF	0.1	(6)
			1,2,3,4,6,7,8-HpCDF	0.01	(<0.1)
			1,2,3,4,7,8,9-HpCDF	0.01	(<0.1)
PCBs			OCDF	0.001	(<0.1)
3,3',4,4' (PCB 77)		0.4			
3,3',4,4',5 (PCB 126)		0.5	Other PCDD/F Congeners		
3,3',4,4',5,5' (PCB 169)		<0.1	2,3-dichlorodibenzo-*p*-dioxin		0.3
Aroclor 1254		<0.1	2,3,7-trichlorodibenzo-*p*-dioxin		37
2,2',4,4',5 (PCB 153)		<0.1	1,2,3,4-TCDD		<0.1

Table 2. Detergent tolerance of the EIA. Aliquots (10 μL) of Triton X-100 in methanol were added to EIA tubes immediately before standards. The critical micelle concentration is 150 ppm[8].

equivalent ppm Triton X-100 in 10 μL sample	2	20	200	2000	10,000
final ppm Triton X-100 in EIA tube	0.04	0.4	4	40	200
I_{50} for 2,3,7,8-TCDD	21	22	20	27	39

Table 3. EIA detection of 2,3,7,8-TCDD spiked into crude soil extracts. The effective loading of the soil matrix was 10 mg per EIA tube. The concentration of 2,3,7,8-TCDD added was 10 pg/EIA tube. This corresponds to an original soil concentration of 10 pg/10 mg or 1 ng/g.

Sample	solvent blank	extraction blank	soil 1	soil 2	soil 3
pg/tube detected in unspiked extract	1.4	1.1	4.5	5.0	4.8
pg/tube detected in spiked extract	10.5	10.8	10.8	11.1	9.5
blank subtracted pg/tube detected in spiked extract	9.1	9.7	6.3	6.1	4.7
blank corrected percent recovery of spike	100	107	69	67	51

Figure 1. Sensitivity and reproducibility of the dioxin immunoassay. Response data from 18 runs over 5 weeks are plotted as mean±SD (n = 7 to 18 for individual points). The 10 pg/tube standard (response of 65%) represents a 10 μL aliquot of a 1 ng/mL solution. This corresponds to the concentration of 2,3,7,8-TCDD in Calibrator 1 for EPA Method 8290[6], indicating sensitivity competitive with HRMS methods.

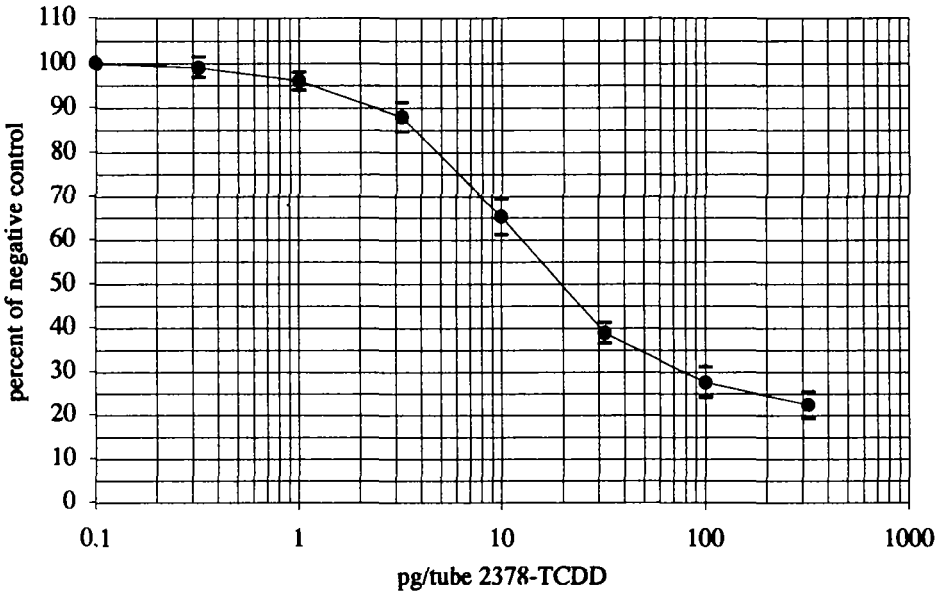

Figure 2. Validation of TEQ measurement concept for 20 fish samples. EIA response (predicted by additive response model) plotted against actual TEQ (determined by GC-MS). The correlation between predicted EIA and TEQ was 0.987, indicating excellent predictive power of the EIA.

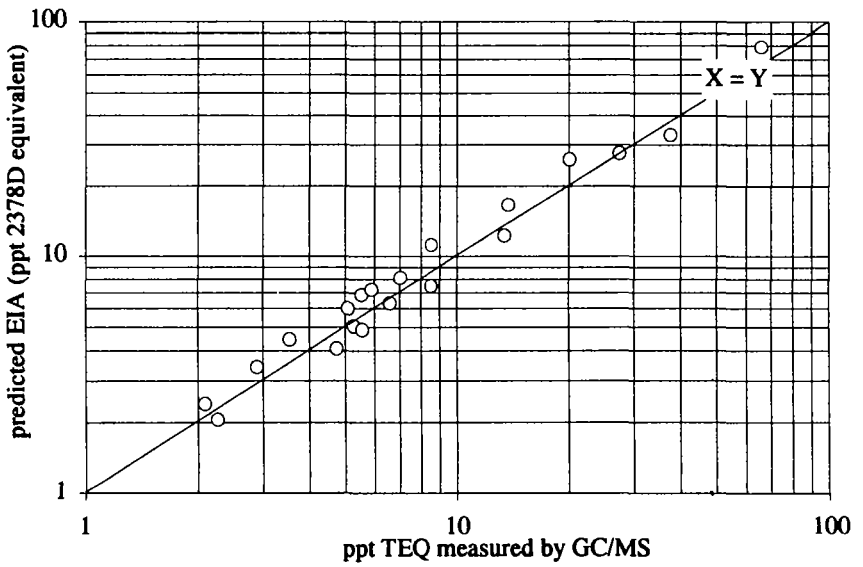

Figure 3. Possible PCDD/F Immunoassay Screening Strategy. This strategy is modeled after Sherry (1) and requires the preparation of only one extract. The strategy is made possible by the use of mass-labeled internal standards which are EIA compatible. Subsamples can be removed for EIA analysis at a variety of points in the conventional cleanup process, avoiding significant portions of the cost for conventional analysis.