Rapid Dioxin Screening by Enzyme Immunoassay

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ABSTRACT

A system has been developed for rapid screening of 2,3,7,8-TetraChloroDibenzo-p-Dioxin (TCDD). The system uses a competitive inhibition Enzyme ImmunoAssay (EIA) based on a mouse monoclonal antibody which is specific for TCDD and related congeners. Sample preparation can be performed with a programmable automated extraction and cleanup system which uses disposable Teflon clad columns. The extraction and cleanup system has been extensively validated by GC-MS for a variety of sample types. The sample preparation system allows immunoassay analysis of soil, serum, water, and other matrices by taking each sample type to the same sample preparation endpoint. Concentration factors and endpoint conditions are completely flexible and programmable. Immunoassay analysis is performed by the addition of a prepared sample extract in organic solvent to an antibody coated microwell containing an aqueous sample diluent. This is mixed and incubated for 30 minutes to allow the immobilized antibody to capture analyte from the sample. The liquid is then removed and the well is washed to remove unbound materials. The well is then incubated with a competitor-HRP conjugate capable of binding specifically to the antibody sites not occupied by TCDD. After 30 minutes, the unbound conjugate is washed away and enzyme substrate is added for color development. The color generated is directly related to the amount of competitor-HRP bound in the second step, which is inversely related to the amount of analyte bound in the first step. After 30 minutes, a stop solution is added and the developed color is read on a microplate reader. The total time required for the EIA analysis of a prepared extract is less than 2 hours. Sensitivity for TCDD is better than 0.1 ng/well, allowing sensitive analysis of a variety of environmental matrices. Preliminary results indicate that it is possible to detect 10 ppb 2378-TCDD in soil by direct analysis of crude soil extracts. Work is being directed toward simplification of the extraction procedure and improvement of the interface with the automated sample cleanup system. The data presented here demonstrate that this system should be useful for TCDD screening in many situations for a variety of matrices. The system offers significant improvements in speed, sample throughput, and cost compared to GC-MS.

INTRODUCTION

Reagents and Standards

The EIA for PCDD/F's uses the mouse monoclonal antibody DD3, which has been described previously[1]. Competitors which bind specifically to the dioxin binding site of DD3 were conjugated to horseradish peroxidase (HRP) to make conjugates which can be captured by the immobilized DD3 antibody. The competitor-HRP conjugates were tested for PCDD/F sensitivity and solvent and matrix tolerance. Standard preparation was as follows, based in part on prior work by Sherry et al.[2] PCDD/F standards in toluene or nonane were diluted in

the same solvent in silanized glass vials. A small volume of DMSO was added to each vial, then the toluene or nonane was evaporated under a nitrogen stream at 40-50°C. The DMSO was diluted with an equal volume of methanol and the standard was mixed vigorously, then sonicated for 15 minutes. Analysis was as described in the EIA procedure below, by adding the DMSO/methanol solution to aqueous diluent in the EIA well.

PCDD/F EIA Procedure and Interpretation of Results

Following is the procedure for the EIA analysis of PCDD/F's: 1) mouse antibodies which recognize the dioxin structure are immobilized on the walls of plastic microwells; 2) PCDD/F's in solvent, in the form of either standards or samples prepared as described above, are mixed with Assay Diluent in the wells, allowing PCDD/F's to bind to the immobilized antibodies; 3) unbound sample is washed away with water; 4) competitor-HRP conjugate is added and allowed to compete with the captured analyte for the limited PCDD/F binding sites on the immobilized antibodies; 5) unbound conjugate is washed away with water, leaving an amount of conjugate on the immobilized antibodies inversely related to the amount of PCDD/F's that were present in the sample; 6) enzyme substrate is added to the wells for color development by the bound enzyme. The intensity of color is proportional to the amount of bound enzyme and is inversely related to the amount of PCDD/F's present in the sample. Therefore, ***more color means less PCDD/F's***. Total run time is approximately 2 hours per test and up to 40 samples can be run in a single batch. The optical density (OD) of each standard and sample well is measured and sample PCDD/F concentrations are calculated based on the standard curve.

RESULTS AND DISCUSSION

Method Sensitivity

The use of heterologous haptens or competitors for improving immunoassay sensitivity has been described previously in detail[3] and has been exploited here using a well studied anti-dioxin antibody. Three heterologous competitors were compared to the homologous competitor. The results of a single experiment (Figure 1) show that the improvement in sensitivity obtained was greater than an order of magnitude for one competitor and approximately one order of magnitude for the other two competitors. Conjugates 1c and 2a were selected for further characterization. Sensitivity to 2378-TCDD in all subsequent experiments with both 1c and 2a has been better than 100 pg/well.

Test Specificity

The specificity of DD3 antibody has been described previously[1] and is primarily directed toward selected tetra- and pentachlorodibenzodioxins, with reduced recognition of the corresponding furans. This recognition profile corresponds roughly to published international toxic equivalency (I-TEF) values[4]. Competitive inhibition tests were performed using a native standard mixture to assess the possibility of a change in specificity due to the use of heterologous competitors. The mixture consisted of the 7 dioxins and 10 furans containing at least the 2,3,7,8 chlorination pattern. This mixture contains compounds comprising the full range of recognition by DD3 antibody in the system used by Stanker et al.[1], as well as TEF values covering three orders of magnitude[4]. The ratio between mass concentration and toxic equivalent concentration (TEC) of the mixture was 8.68. The results shown in Figure 2 suggest that the specificity of DD3 antibody with competitor 1c does not differ significantly from the previously established pattern. Similar results were seen for competitor 2a.

Interface of EIA with Automated Sample Cleanup System

This EIA system is capable of analyzing samples in a variety of solvents, but has been designed to accommodate any sample that can be exchanged from a volatile hydrophobic solvent into a non-volatile hydrophilic solvent. This allows the analysis of any sample

prepared by standard methods such as the FMS Dioxin-Prep™ System for Automated Sample Cleanup[5]. Preliminary experiments indicate no interference in the EIA using fully cleaned soil or serum extracts from the FMS system. Ultimate method sensitivity is therefore determined primarily by sample size, concentration factor, and interference from the concentrated matrix.

Soil Spiking and Extraction

The possibility of a rapid extraction and analysis for PCDD/F's in soil was investigated in two experiments performed as follows. In the first experiment, aliquots of 5 g of soil were weighed into silanized glass extraction vials and air dried overnight. Soils were spiked by adding a toluene solution of 2378-TCDD directly to the soil surface at multiple sites. After mixing the soil and air drying 30 minutes, 3 steel BB's and 5 ml of solvent were added to each vial. Dichloromethane and toluene samples were prewetted with a minimum amount of acetone before adding the other solvent. Vials were sealed with Teflon lined caps and soil samples were extracted by shaking for thirty minutes at 300 rpm on an orbital shaker. The extracts were clarified by centrifugation for 15 minutes at 1-2000g. An aliquot of each extract was removed to a silanized vial, DMSO was added, and the volatile solvents were removed by evaporation under a nitrogen stream with heating to 40-50°C. Each sample was diluted with an equal volume of methanol and further handling and analysis followed the procedure described above for standards. The data of Table 1 show that 2378-TCDD spikes may be recovered and detected with a relatively simple procedure, but that not all solvents will give adequate results. Toluene appears to extract soil components which give strong false positive interference in the EIA, while DMSO appears to give inadequate recovery. Neither dichloromethane nor hexane:acetone gave significant false positive interference and both gave acceptable recovery values.

The same procedure was followed for the second experiment, except for the following changes. Four spike concentrations were used, soils were air dried overnight after spiking, a larger volume of extract was concentrated into DMSO for EIA analysis, and two different sample volumes were used for the EIA. The matrix effect for hexane:acetone was less than for dichloromethane at both sample volumes (Table 2). The sensitivity to 2378-TCDD appeared better at the lower sample volume. Recoveries of spiked standard appeared incomplete for both extraction methods, suggesting that much tighter binding to soil may occur during the overnight drying step than during the 30 minute drying step of the first experiment. Despite this reduced recovery, the increase in sample concentration factor allowed the 10 ppb spike to be detected easily. These results will form the basis of further experiments directed toward a rapid soil extraction procedure for low level analysis of PCDD/F's in soil.

CONCLUSIONS

1. The test is capable of analyzing for PCDD/F's in less than 2 hours from prepared extracts, using very little specialized equipment.
2. The heterologous competitor strategy employed here demonstrates significantly improved sensitivity.
3. The specificity of the test appears to parallel the previously established profile for DD3 antibody.
4. The design of the EIA accommodates significant variations in sample preparation, allowing the analysis of PCDD/F's in many matrices.
5. Ongoing work with this kit includes improvement of the rapid soil extraction procedure and validation for a variety of sample matrices.

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TABLES AND FIGURES

Table 1. Recovery of 2378-TCDD from Soil by Four Extraction Methods. One soil was spiked at 10 ng/g with 2378-TCDD in toluene and recovery was compared to a toluene standard evaporated from a silanized vial and recovered with DMSO. Each extraction method and the no soil control included an unspiked sample for evaluation of the matrix effect. Each value is the mean for three replicate wells in one EIA run.

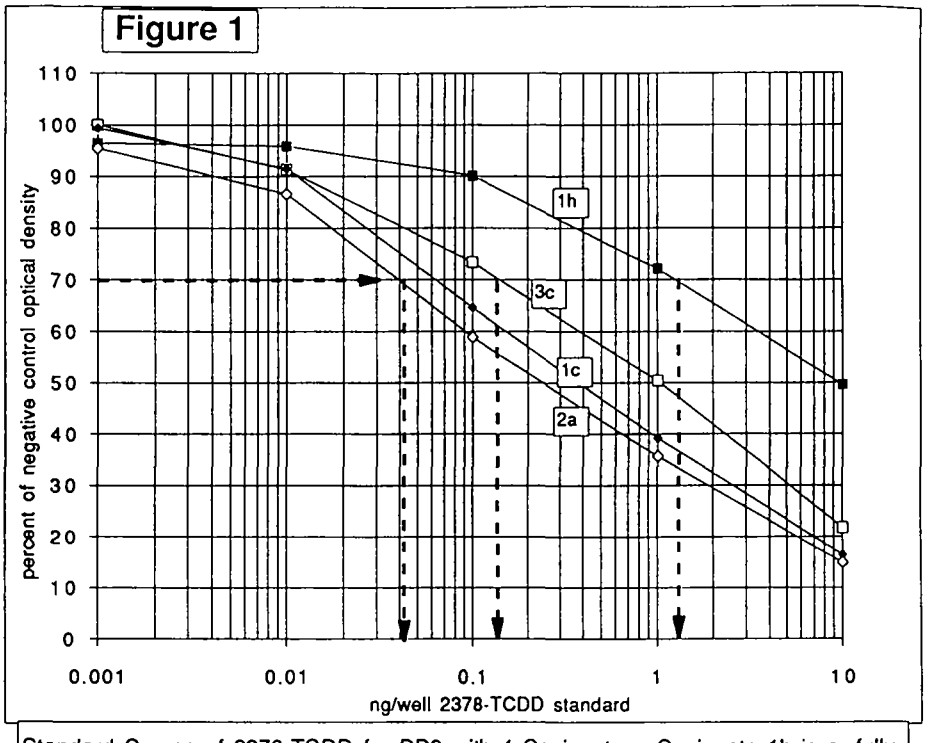
Extraction Solvent	Matrix Effect*	Recovery of Standard**
DMSO	92	36
toluene	56	167
dichloromethane	108	91
hexane:acetone (1:1)	120	94

* Optical density (OD) of unspiked soil as a percent of the no soil/no TCDD control OD in the EIA; decreased OD indicates false positive interference.
** Percent recovery of 10 ng/ml soil spike relative to spike recovered from vial with no soil.

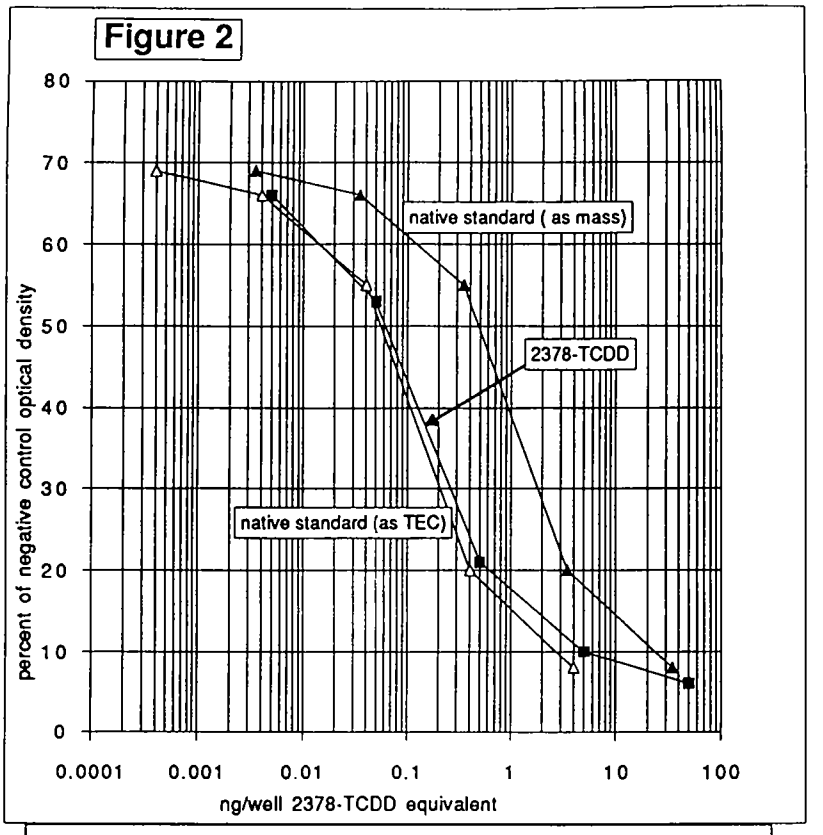
Table 2. Recovery of 2378-TCDD from Soil by Two Extraction Methods. One air dried soil was split and spiked at 0, 1, 10, and 100 ng/g with 2378-TCDD in 50 µl toluene. Each extract was compared to the same spiking solution evaporated onto a bed of DMSO in a silanized vial. Each value is the mean for three replicate wells in one EIA run.

	µl sample	Matrix	Recovery of Standard**		
Extraction Solvent	used in EIA	Effect*	1 ppb	10 ppb	100 ppb
no soil control	10	—	55	25	20
dichloromethane	10	93	94	66	38
hexane:acetone (1:1)	10	123	90	62	26
no soil control	50	—	28	18	21
dichloromethane	50	45	153	96	66
hexane:acetone (1:1)	50	86	89	64	32

* Optical density (OD) of unspiked soil as a percent of the no soil/no TCDD control OD in the EIA; decreased OD indicates false positive interference.
** Optical density (OD) of standard or spiked soil as a percent of the 0 ppb standard or soil OD in the EIA; soil OD higher than standard OD indicates incomplete spike recovery.



Standard Curves of 2378-TCDD for DD3 with 4 Conjugates. Conjugate 1h is a fully homologous system, where the competitor used for HRP conjugation is the same as the haten used for developing the antibody. The other three conjugates use competitors for HRP conjugation which are different than the hapten used for developing the antibody.



Comparison of EIA Standard Curves of 2378-TCDD and a 17 Congener Native Standard Mixture. HRP conjugate 1c and DD3 antibody were used to detect both 2378-TCDD and native standard (Table 1). The native standard response is expressed both as actual total mass and as toxic equivalent concentration (TEC) according to Table 1.