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THE USE OF RADIOIMMUNOASSAY FOR THE DETECTION OF POLYCHLORINATED DIBENZO-P-DIOXINS IN FISH SAMPLES

by

J. Sherry[1], J. ApSimon[2], L. Collier[2],
R. Wilkinson[1], P. Albro[3] and B. Afghan[4]

[2] Ottawa Carleton Chemistry Institute,
Carleton University
Ottawa, Ontario K1S 5B6

[1] Research and Applications Branch
National Water Research Institute,
867 Lakeshore Road, P.O. Box 5050
Burlington, Ontario, Canada
L7R 4A6

[3] National Institute of Environmental Health Sciences
North Carolina, U.S.A.

[4] National Water Quality Laboratory
Burlington, Ontario, Canada

MANAGEMENT PERSPECTIVE

The timely and cost-effective analysis of polychlorinated dibenzo-p-dioxins (PCDDs) is of a high priority. Conventional methods for the determination of PCDDs in environmental samples are time consuming and expensive. The employment of a screening test will, through the elimination of PCDD, free samples from further analysis, and increase overall analytical efficiency. The radioimmunoassay (RIA) for PCDDs was developed to satisfy an IWD requirement for such a screening capability.

The RIA for the detection of PCDDs was interfaced with an extraction and cleanup procedure, and its performance was assessed using extensively and minimally cleaned-up Lake Trout samples. Sample size appeared to influence assay performance, probably because of its relationship to the specific detection limit: the larger the sample size that can be analyzed without adversely affecting the amount of TCDD detectable, the lower will be the specific detection limit. However, larger than optimal samples narrowed the assay's working range, adversely affected dose response, and raised the detection limit. The working range of the assay results from a compromise between the required degree of cleanup and sample size.

The RIA for PCDDs provides analysts with a tool to screen environmental samples for the presence of PCDDs. RIA can also be used to rapidly confirm the results of GC/MS analyses. The RIA for PCDDs should prove useful in environmental surveillance and monitoring programs, as well as in the analysis of other large sample sets.

Dr. J. Lawrence
Director
Research and Applications Branch

PERSPECTIVE DE GESTION

Il est urgent de mettre au point une analyse rapide et efficace des dibenzo-p-dioxines polychlorés (PCDD). Les méthodes classiques de dosage des PCDD dans les échantillons environnementaux prennent beaucoup de temps et coûtent cher. L'utilisation d'un test d'isolement sélectif pourrait, grâce à l'élimination des PCDD, éviter d'avoir à soumettre les échantillons à d'autres analyses et augmenter l'efficacité globale des analyses. Un radio-immunoessai (RIA) pour le dosage des PCDD a été mis au point à la demande de la DEI qui avait besoin d'une telle capacité d'isolement sélectif.

Le RIA pour le dosage des PCDD a été relié à une méthode d'extraction et de nettoyage et sa performance a été évaluée à l'aide d'échantillons de truite de lac nettoyés à fond et à peine. Il est apparu que la taille des échantillons influait sur la performance de l'essai, plobablement à cause de son rapport avec la limite de détection spécifique : plus la taille de l'échantillon qui peut être analysée sans que cela affecte négativement la quantité de PCDD détectable est importante, et plus la limite de détection spécifique sera basse. Toutefois, les échantillons dont la taille dépasse la taille optimale ont rétréci la gamme de travail de l'essai, perturbé la dose réponse et haussé la limite de détection. La gamme de travail de l'essai découle d'un compromis entre le degré nécessaire de nettoyage et la taille de l'échantillon.

Le RIA mis au point pour le dosage des PCDD permet aux analystes de détecter la présence de ces contaminants dans les échantillons environnementaux. Cette méthode peut également être utilisée pour confirmer rapidement les résultats des analyses par chromatographie gazeuse/spectrométrie de masse. Le RIA utilisé pour doser les PCDD devrait se révéler utile dans les programmes de surveillance et de contrôle de l'environnement, ainsi que dans l'analyse des autres grandes séries d'échantillons.

Dr. J. Lawrence
Directeur
Direction de la recherche pure et appliquée

ABSTRACT

Because of the increasing numbers of environmental samples requiring analysis for polychlorinated dibenzo-p-dioxin (PCDD) contamination, a need exists for screening techniques, such as radioimmunoassay (RIA), that will facilitate the elimination of PCDD free samples from time-consuming conventional analysis. The RIA for the detection of PCDDs was interfaced with an extraction and cleanup procedure, and its performance was assessed using extensively and minimally cleaned-up Lake Trout samples. Sample size appeared to influence assay performance, probably because of its relationship to the specific detection limit: the larger the sample size that can be analyzed without adversely affecting the amount of TCDD detectable, the lower will be the specific detection limit. However, larger than optimal samples narrowed the assay's working range, adversely affected dose response, and raised the detection limit. The working range of the assay results from a compromise between the required degree of cleanup and sample size.

KEYWORDS

Radioimmunoassay; polychlorinated dibenzo-p-dioxins; fish; detection; screening; cleanup.

RÉSUMÉ

À cause du nombre sans cesse croissant d'échantillons environnementaux qui doivent être analysés pour détecter la contamination par les dibenzo-p-dioxine polychlorées (PCDD), il est nécessaire de mettre au point des méthodes d'isolement sélectif, comme le radio-immunoessai (RIA), qui permettent d'éliminer rapidement les échantillons exempts de PCDD des longues analyses classiques. Le RIA utilisé pour doser les PCDD a été relié à une méthode d'extraction et de nettoyage et sa performance a été évaluée à partir d'échantillons de truite de lac nettoyés à fond et à peine. La taille des échantillons semble influer sur la performance de l'essai, probablement à cause de son rapport à la limite de détection spécifique : plus la taille de l'échantillon qui peut être analysée sans perturber la quantité de TCDD détectable est importante, et plus la limite de détection spécifique est basse. Toutefois, les échantillons dans la taille est supérieure à la taille optimale rétrécissent la gamme de travail de l'essai et perturbent la dose réponse tout en augmentant la limite de détection. La gamme de travail de l'essai découle d'un compromis entre le degré de nettoyage nécessaire et la taille de l'échantillon.

1.0 INTRODUCTION

Environmental persistence, exceptionally high toxicity, and accumulation in the food chain have made the timely and cost effective analysis of the polychlorinated dibenzo-p-dioxins (PCDDs) a high priority (NRCC, 1981). Conventional methods for the determination of PCDDs in environmental samples are time consuming and expensive: a single analysis, with isomer confirmation, can cost in excess of $1500. Increased public concern coupled with a growing scientific desire to determine the sources, locations, and fate of PCDDs in the environment has resulted in increased sample loads for analytical laboratories. Such demands are unlikely to diminish in the future. A screening test would help solve such difficulties by eliminating PCDD free samples from further analysis. Positive samples from the screening step would have to undergo confirmatory analysis using gas chromatography-mass spectrometry.

Based on the classical antigen-antibody reaction, radioimmunoassay (RIA) is a relatively simple, powerful, and adaptable technique for the rapid determination of trace levels of organic compounds (McCormick and Schmitz, 1984). Antibodies produced against an analyte are incubated in a competitive binding reaction with labelled (usually ^{125}I) and unlabelled analyte. A reduction in antibody binding of the labelled analyte is inversely proportional to the quantity of unlabelled analyte present. The amount of analyte in samples is interpolated from a standard curve.

Originally developed by Albro and co-workers (Albro et al., 1979), the RIA for the detection of PCDDs was evaluated and modified in our laboratories (Sherry et al., 1988), for eventual use in the detection of PCDDs in aquatic environmental samples. When evaluated using 2,3,7,8-TCDD standards, the modified assay, which uses dimethyl sulfoxide as the dioxin solubilization agent, was fast (overnight detection), sensitive (the precision of measurement of zero dose was determined to be 6 pg of 2,3,7,8-TCDD), and had a minimum detectable

concentration (MDC: the estimated amount of analyte present when the response of the assay calibration curve at zero concentration was added to three times the replicate adjusted error in that response) of 15 pg of 2,3,7,8-TCDD.

Our next objective was to interface the RIA with an extraction and cleanup procedure suitable for use with fish: a matrix that is commonly analyzed for PCDD contamination. The sample preparation procedure selected for use with the RIA had been previously developed (Afghan et al., 1987) for the conventional analysis of a variety of matrices; it was hoped, if possible, to employ a simplified version of this multi-step procedure. To be useful in the screening of fish samples for 2,3,7,8-TCDD contamination, a screening test should be able to detect low ppt levels of analyte. Consequently, it was important to investigate the effect of sample size and the degree of sample cleanup on assay performance: sample size could influence the specific detection limit and is directly related to assay capacity.

2.0 MATERIALS AND METHODS

The antiserum used (ALB 5) was prepared by P. Albro of the NIEHS, USA. The currently used hapten is 1-N-(5-iodovaleramido)-3,7,8-trichlorodibenzo-p-dioxin, in which the iodo group is displaced by ^{125}I.

2.1 DMSO Assay

The DMSO based assay uses 33.3% (v/v) DMSO to solubilize PCDDs. Ultrasonication (30 min) is used to assist solubilization. Antibody and sample are pre-incubated before additon of the tracer (7000 cpm). After overnight incubation at 4°C, bound and unbound tracer are separated using a polyethylene glycol assisted second antibody procedure.

2.2 Triton Assay

The Triton based assay uses .45% (w/v) Triton X-305 to solubilize PCDDs. Ultrasonication (30 min) is used to assist solubilization. Antibody and sample are pre-incubated before addition of the tracer. After 64 hr incubation, bound and unbound tracer are separated as described for the DMSO based assay.

2.3 Fish

A Lake Trout (Salvelinus namaycush), taken from Jackson Lake, Wyoming after a virus kill was homogenized whole, sub-sampled, and stored at -20°C until use. The fish was extracted in 10 g portions by means of overnight agitation in HCl:toluene (1:1). Three concentrations of HCl were used in the extraction: N, 6N, and 10N (Table 1). The cleanup combinations used are described in Table 1. A spiked sample that was processed along with the RIA samples yielded 93% recovery of a 2,3,7,8-TCDD^{13}C spike. The following native homologues were tentatively identified: pentachloro-DBD (0.48 pg/100 mg), heptachloro-DBD (3.8 pg/100 mg), and octrachloro-DBD (25 pg/100 mg). The penta- and heptachloro-DBD levels are below the RIA's detection limit for those homologues, and ALB 5 did not cross-react significantly with octachloro-DBD in our assay.

Fish extracts (in toluene) were spiked with unlabelled 2,3,7,8-TCDD (KOR) immediately prior to RIA analysis. A sample was considered positive if it was distinguishable from a matrix blank using Student's t test (n = 3 or 5).

3.0 RESULTS

Because a low (10) to mid (500) ppt detection range for 2,3,7,8-TCDD was considered desirable, and with the intention of keeping the number of cleanup steps to a minimum, the effect of both sample

size, and the degree of cleanup on assay performance was investigated. Since preliminary results had indicated that assay capacity could be a problem with the DMSO based assay, the Triton based assay was also included in these experiments.

The data in Table 2 indicate that the extent of sample cleanup affected assay capacity. Of the cleanup combinations (Table 1) examined, those that did not incorporate the alumina chromatography step resulted in assay overload with the 300 mg samples. No assay overload was observed, even with the 1200 mg samples, when the complete cleanup procedure was used.

The Triton assay underwent smaller overload effects (maximum negative shift of 300-400 cpm, relative to the control tubes) than the DMSO assay (maximum positive shift of 500-1100 cpm, relative to the control tubes). The Triton assay demonstrated superior capacity to the DMSO assay with the F9(6N,GPC,AL) and F4(GPC,H^+,AL) cleanup combinations. Assay overload may be caused by saturation of the dioxin solubilization agent with hydrophobic materials and resultant precipitation of labelled dioxin; such an occurrence probably caused the overload effect that was observed with the DMSO assay. Preferential solubilization of the labelled hapten by matrix components could shield the hapten from the antibody binding sites, resulting in a decrease in the amount of bound radioactivity relative to the control tubes, as was observed in the case of the Triton assay.

The extent of cleanup and sample size also affected the ability of the assay to detect a 300 pg 2,3,7,8-TCDD spike. It was possible to detect the spike in the presence of 600 mg of sample matrix with the F5(GPC,TRIP,H^+,Al) and F7(complete) cleanup combinations; the largest response was obtained with the F7(complete) combination. The Triton assay was not tested for spike detection with the 300 and 600 mg samples owing to a shortage of sample extracts. Future cost considerations prompted an investigation into the possibility of eliminating some of the cleanup steps. The data in Tables 2 and 3, indicated that the F5(GPC,TRIP,H^+Al) cleanup combination offered the best prospects for a

reduced cleanup combination based on the method currently in use at NWRI. The F9(6N,GPC,AL) combination was also selected for investigation in order to examine assay performance in the presence of minimally cleaned extract. The complete cleanup combination was reserved for future evaluation should the reduced cleanup systems yielded unsatisfactory results.

The performance of the RIA with minimally cleaned samples is documented in Table 4; the lowest concentration of 2,3,7,8-TCDD detected was 210 ppt, using the Triton assay. Increased sample size narrowed the working range of the assay from 620 to at least 820 ppt in the case of the DMSO assay. Probably because of the increased capacity of the Triton assay, increased sample size caused an apparent improvement in the Triton assay's working range, although the Triton assay was not tested at less than 31.25 pg with the 100 mg sample. Extensively cleaned (GPC,TRIP,H^+,Al) sample extract was used in subsequent experiments.

A wide range of spikes was detected in the extensively cleaned extract using both the DMSO (Table 5a) and Triton (Table 5b) assays. For each assay the range of spikes detected was affected by sample size. A dose response was obtained for the 100 mg samples, but not for the 300 mg samples, which indicated the presence of residual matrix interferences. Increasing the sample size to 600 mg narrowed the working range of the Triton assay.

Table 6 indicates that the least amount (pg) of 2,3,7,8-TCDD detected in the presence of the extensively cleaned extract was affected by sample size and the type of dioxin solubilization system used: lower amounts of 2,3,7,8-TCDD being detected in the 100 mg samples. However, approximately equal (DMSO) (note: the DMSO assay was not tested at the 133 ppt level) or apparently lower (Triton) LCDs (least concentration detected) were obtained for the 300 mg samples. A lower LCD was obtained for the Triton (67 ppt) than the DMSO (200 ppt) assay.

4.0 CONCLUSIONS

The described experiments demonstrated that the quantity of sample analyzable using RIA was dependent on the extent of sample preparation. The extent of sample cleanup also affected the ability of the RIA to detect a 2,3,7,8-TCDD spike in fish matrix. The minimum cleanup level necessary for the detection of a 300 pg TCDD spike in the presence of 300 mg of fish matrix yielded unsatisfactory LCDs using both the Triton and DMSO based assays. These observations are a consequence of a key problem associated with immunoassays for PCDDs: dioxins are highly insoluble in aqueous solution, yet for an immunoassay to be successful a mechanism must be devised to render PCDDs soluble and accessible for antibody binding; such a mechanism must not cause antibody denaturation or overly interfere with assay sensitivity. The versions of the RIA evaluated in the present study use DMSO or the non-ionic surfactant Triton X-305 to solubilize PCDDs. Both solubilization systems were found to be prone to overloading effects caused by matrix interferences: the Triton assay being less susceptible than the DMSO assay. Furthermore, the presence of residual hydrophobic materials in the partially cleaned sample extracts could also solubilize 2,3,7,8-TCDD molecules and shield them from the antibodies, thus reducing assay sensitivity.

Lower LCDs were obtained using the extensively cleaned fish matrix. In contast to the results obtained using 2,3,7,8-TCDD standards in the absence of sample matrix, the Triton based assay yielded lower LCDs than the DMSO assay, probably because of the greater matrix capacity of the Triton assay. Increasing the sample size beyond an optimal level raised the detection limit and adversely affected dose response.

The inclusion of a further cleanup step may help to achieve the desirable objective of lowering the RIA's specific detection limit to the region of 20 ppt, by allowing a further increase in sample size whilst lowering the assay's detection limit. With this goal, and

considering the results of the capacity and spike detection experiments, the complete cleanup combination (GPC,TRIP,H^+,AL,CF) shall be further evaluated. Modifications to the sample preparation procedure could reduce the number and duration of the extraction and of cleanup steps required for RIA. While inclusion of the carbon chromatography step may allow us to dispense with the alumina chromatography step. Although such an extensive cleanup protocol would offer no savings in sample preparation time compared to conventional analytical methods, unless it should prove possible to eliminate one or other of the post GPC steps, RIA could still be used to reduce the sample load on high resolution mass spectrometers. Other matrices may not require as extensive a cleanup as Lake Trout, or, if heavily contaminated, as is the case with some industrial samples, may not demand as low a detection limit, in which case the extent of sample preparation could probably be reduced.

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TABLE 1

Preparation of Fish Extracts

Sample Number	Cleanup Step				
	GPC[1]	TRINaP[2]	H_2SO_4[3]	ALUMINA[4]	CF[5]
1	+*				
2	+	+			
3	+	+	+		
4	+		+	+	
5	+	+	+	+	
6	+			+	
7	+	+	+	+	x
9(6N[6])	+			+	
10(10N[7])	+			+	

*: Cleanup step was used.

[1] GPC: Gel Permeation Chromatography (Waters Styragel column) for bulk lipid removal.

[2] TRINaP: Trisodium phosphate wash for removal of phenolics, acidic compounds, and sulphur compounds

[3] H_2SO_4: Acid wash for removal of residual lipids and basic compounds

[4] ALUMINA: Aluminum oxide (basic) chromatography for removal of PCBs, PAHs, trace phenolics, and DDE

[5] CF: Carbon fibre chromatography (Amoco PX 21) for removal of neutral pesticides and non planar organic contaminants

[6] Sample extracted using 6N HCl

[7] Sample extracted using 10N HCl

TABLE 2

Capacity of RIA Using Various Extraction and Cleanup Combinations

Sample Number	Sample Size (mg per tube) 300	600	1200
		(a) DMSO Assay	
F1 (GPC)	+		
F2 (GPC,TRIP)	+		
F3 (GPC,TRIP,H^+)	+		
F4 (GPC,H^+,AL)	-	+	++
F5 (GPC,TRIP,H^+,AL)	-	-	++
F6 (N,GPC,AL)[1]	-	-	++
F7 (GPC,TRIP,H^+,AL,CF)	-	-	-
F9 (6N,GPC,AL)	-	+	++
F10 (10N,GPC,AL)	-	+	++
		(b) TRITON Assay	
F2 (GPC,TRIP)	+	+	+
F3 (GPC,TRIP,H^+)	+	+	+
F4 (GPC,H^+,AL)	-	-	++
F5 (GPC,TRIP,H^+,AL)	-	-	++
F6 (N,GPC,AL)[1]	-	-	-
F7 (GPC,TRIP,H^+,AL,CF)	-	-	-
F9 (6N,GPC,AL)	-	-	+
F10 (10N,GPC,AL)	-	+	+

-: No overload

+: Overload

++: Gross overload

1: Precipitate formed in hexane:dcm

TABLE 3

Ability to Detect a 2,3,7,8-TCDD Spike (300 pg) in Fish Matrix

	Solubilization System			
	DMSO sample size (mg per tube)			TRITON
	100	300	600	100
F1 (GPC)	-	nd	nd	-
F2 (GPC,TRIP)	-	nd	nd	+
F3 (GPC,TRIP,H^+)	+	ol	ol	+
F4 (GPC,H^+,AL)	+	+	ol	+
F5 (GPC,TRIP,H^+,AL)	+	+	+	+
F6 (N,GPC,AL)	+	+	-	+
F7 (GPC,TRIP,H^+,AL,CF)	+	+	+	+
F9 (6N,GPC,AL)	+	+	ol	+
F10 (10N,GPC,AL)	+	-	ol	+

ol: overload
nd: not done
+: spike detected
-: spike not detected

TABLE 4

Ability to Detect a Range of 2,3,7,8-TCDD Spikes:
Minimal Extract Cleanup (6N,GPC,AL).

Spike (pg)	Sample Size (mg per tube)			
	100		300	
	(a) DMSO Assay			
31	NS		NS	
63	+***	(630 ppt)	NS	
125	+***		NS	
250	+***		NS	
	(b) TRITON Assay			
31	+**	(310 ppt)	NS	
63	+*		+***	(210 ppt)
125	+***		+*	
250	+*		+*	

NS: No significant difference (P 0.05) detected between the spiked sample and the matrix blank

+: Spike detected

*: P 0.05

**: P 0.01

***: P 0.005

TABLE 5

Ability to Detect a Range of 2,3,7,8-TCDD Spikes:
Extensive Cleanup (6N,GPC,TRIP,H^+,AL).

Spike (pg)	Sample Size (mg per tube)					
	100		300		600	
	(a) DMSO Assay					
31	+**	(310 ppt)	NS	(100 ppt)		
63	+**	(630 ppt)	+**	(210 ppt)		
125	+**		+**	(420 ppt)		
250	+**		+**			
500	+**		+**			
1000	+**		+**			
	(b) TRITON Assay					
31	+***	(310 ppt)	+***	(100 ppt)	-	
63	+***		+***	(210 ppt)	NS	(105 ppt)
125	+***		+***	(420 ppt)	NS	(210 ppt)
250	+***		+***		-	
500	+***		+***		+***	(835 ppt)
1000	+***		+***		-	

NS: No significant difference (P 0.05) detected between the spiked sample and the matrix blank

+: Spike Detected; *: P 0.05

**: P 0.01 ***: P 0.05

TABLE 6

Least Concentration of 2,3,7,8-TCDD Detected in Fish Matrix: Extensive Cleanup

Spike (pg)	Sample Size (mg per tube)			
	100		300	
	(a) DMSO Assay			
5	NS		NS	
10	NS		NS	
20	+*	(200 ppt)	NS	
31	+*	(310 ppt)	NS	
40	+*		-	
62.5	+**		+**	(210 ppt)
	(b) TRITON Assay			
5	NS		NS	
10	+*	(100 ppt)	NS	
20	+*	(200 ppt)	+*	(67 ppt)
31	+*		+*	

NS: No significant difference (P 0.05) detected between the spiked sample and thematrix blank

+: Spike detected

*: P 0.05

**: P 0.01

***: P 0.005