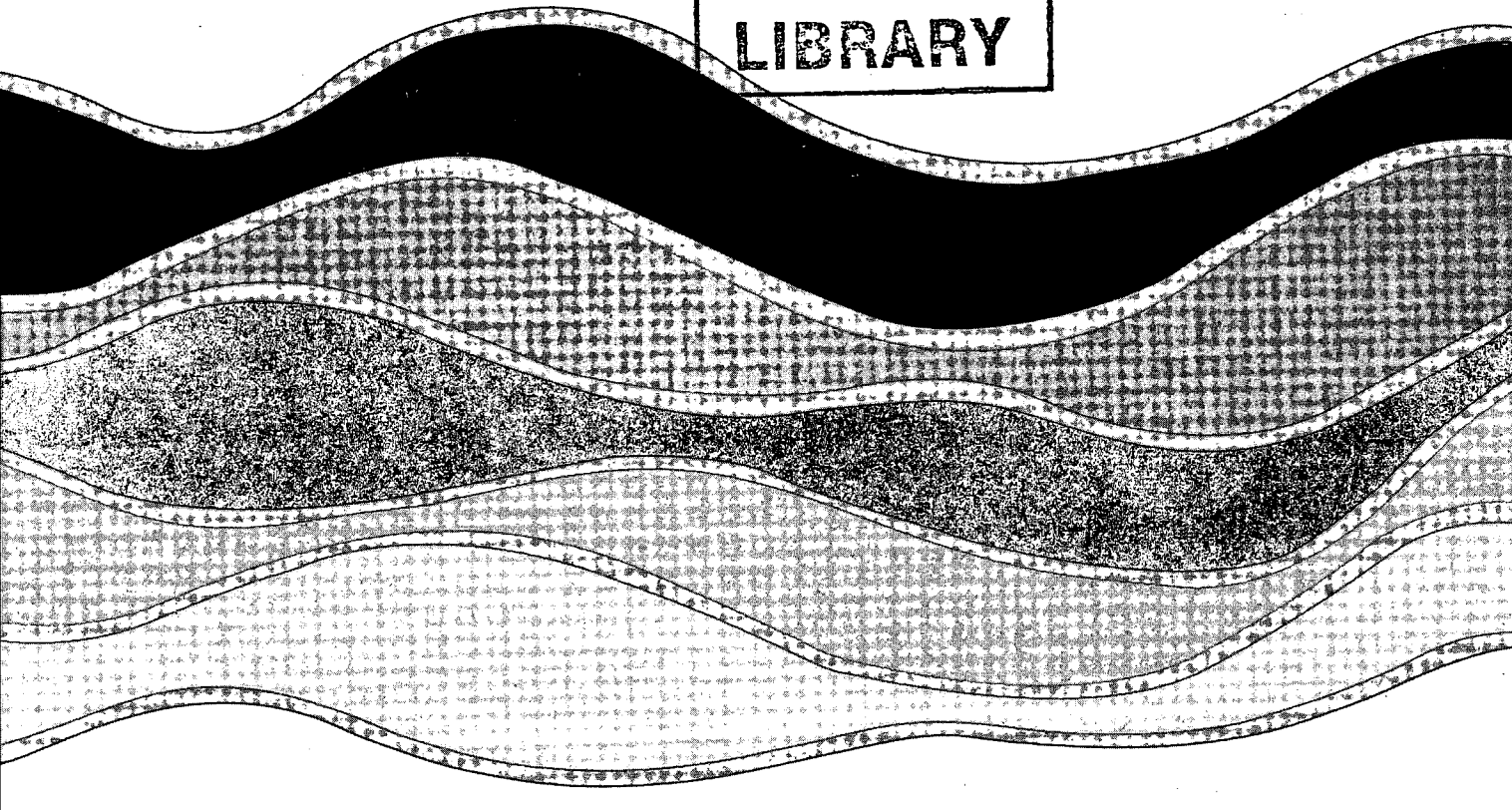
NATIONAL WATER RESEARCH INSTITUTE

INSTITUT NATIONAL de RECHERCHE sur les EAUX



RADIOIMMUNOASSAY FOR THE DETECTION OF POLYCHLORINATED DIBENZO-p-DIOXINS: [^{3}H]-2,3,7,8-TCDD BASED ASSAY VERSION

J.P. Sherry

NWRI Contribution No. 91-128

RADIOIMMUNOASSAY FOR THE DETECTION OF POLYCHLORINATED DIBENZO-p-DIOXINS: [^{3}H]-2,3,7,8-TCDD BASED ASSAY VERSION

J.P. Sherry

Research and Applications Branch
National Water Research Institute
Burlington, Ontario L7R-4A6

NWRI Contribution No. 91-128

MANAGEMENT PERSPECTIVE

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

The version of the method described herein is a product of NWRI project #84017 and has evolved during the course of evaluation, modification, and implementation studies. The sensitivity, detection limit, and speed of the [^{3}H] based assay are superior to the previous [^{125}I] based version of the assay. The method is cost effective ($48 per analysis, excluding sample preparation), rapid, easily learnt, and amenable to automation.

The RIA for PCDDs can be used 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 can be used to screen large sample sets as part of environmental surveillance and monitoring programs.

SOMMAIRE À L'INTENTION DE LA DIRECTION

L'analyse des polychlorodibenzo-p-dioxines (PCDD) par une méthode opportune et rentable est une haute priorité. Les méthodes classiques de dosage des PCDD dans des échantillons prélevés dans l'environnement exigent beaucoup de temps et sont coûteuses. Des épreuves de dépistage, qui permettent de ne pas soumettre les échantillons exempts de PCDD à d'autres analyses, pourraient augmenter l'efficacité analytique générale. Le dosage radio-immunologique modifié pour les PCDD offre à la Direction générale des eaux intérieures une option pour mettre en oeuvre un tel moyen de dépistage.

La version de la méthode décrite ici est le résultat du projet n° 84017 de l'INRE et elle a été élaborée au cours d'études d'évaluation, de modification et de mise en application. La sensibilité, le seuil de détection et la vitesse du dosage au [^{3}H] sont supérieurs à la version antérieure de dosage au [^{125}I]. Il s'agit d'une méthode rentable (48 $ par analyse, en excluant la préparation de l'échantillon), rapide, facile à comprendre, et qui peut être automatisée.

Le dosage radio-immunologique des PCDD peut être utilisé pour déceler la présence de PCDD dans des échantillons prélevés dans l'environnement. Il peut aussi permettre de confirmer rapidement des résultats d'analyses combinant la chromatographie en phase gazeuse et la spectrométrie de masse. Le dosage radio-immunologique des PCDD peut être utilisé pour faire du dépistage parmi le grand nombre d'échantillons recueillis dans le cadre de programmes de surveillance de l'environnement.

ABSTRACT

The radioimmunoassay (RIA) for the detection of polychlorinated dibenzo-p-dioxins (PCDDs) can improve analytical efficiency through the identification of PCDD free samples. The present report describes a modified version of the assay that uses a stable [^{3}H]-2,3,7,8-TCDD radio-ligand and a rapid (5 min) phase separation system that uses activated charcoal. The assay is repeatable, rapid (overnight), and sensitive (the estimated error associated with zero dose is 1.3 pg). A monoclonal antibody based version of the assay is also described. This report is intended as an aid for analysts who wish to implement the technique.

RÉSUMÉ

Le dosage radio-immunologique pour le dépistage des polychlorodibenzo-p-dioxines (PCDD) peut améliorer l'efficacité de l'analyse par la détermination des échantillons exempts de PCDD. On décrit dans le présent rapport une version modifiée de la méthode dans laquelle on utilise un ligand radioactif stable de [^{3}H]-2,3,7,8-TCDD et une séparation de phase rapide (5 min) au charbon activé. Le dosage est répétable, rapide (une nuit), et sensible (l'erreur estimée associée à la dose zéro est de 1,3 pg). On décrit également une version du dosage faisant appel à un anticorps monoclonal. Le présent rapport a été conçu comme un outil pour les analystes qui désirent appliquer la technique.

INTRODUCTION

The chlorinated dibenzo-p-dioxins (PCDDs) are highly toxic contaminants that persist in the environment and tend to accumulate in the food chain. For those reasons the timely and cost effective analysis of PCDDs, particularly the notorious 2,3,7,8-tetrachloro dibenzo-p-dioxin congener (2,3,7,8-TCDD) (1), is a high priority. Conventional methods for the determination of PCDDs are time consuming and expensive: the analysis of a single sample, with isomer confirmation, can cost in excess of $1500. The number and variety of samples that require analysis for PCDDs continues to increase.

The use of a screening test could improve efficiency by identifying PCDD free samples thus facilitating their removal from the analytical process. Immunoassay techniques are widely used in clinical laboratories for the detection and determination of antibodies, hormones, and pharmaceuticals. Since many of these applications involve the detection of trace amounts of organic compounds, it is appropriate to consider the use of immunoassays (IAs) for the detection of trace organic contaminants in environmental matrices.

Originally developed by Albro et al. (2), the radioimmunoassay (RIA) for the detection of PCDDs has been evaluated (3; 4) and modified in our laboratories (5, 6). Assay parameters such as precision, sensitivity, detection limit, repeatability, and specificity have been investigated. The assay has been interfaced with an extraction and cleanup procedure. The previous version of the RIA for PCDDs (7) used an iodinated derivative of 2,3,7,8-TCDD as the radio-ligand. That radio-ligand (^{125}I-valeramido-PCDD) had several shortcomings which adversely affected assay performance and reliability. The unlabelled hapten was unstable: it tended to lose iodine during storage and to cyclize, which resulted in variable yields from the labelling reaction. The low specific activity of the radio-ligand could not be readily increased by an enrichment step. The synthesis

of high activity preparations of [^{3}H]-2,3,7,8-TCDD by ChemSyn (Lenexa, Kansas) provided an alternative radio-ligand (6) that could be used in the RIA. [^{3}H]-TCDD has the advantages of similarity to the target molecule and long shelf life. A sensitive [^{3}H]-2,3,7,8-TCDD based RIA was developed using dextran coated charcoal (DCC) for phase separation (6). The assay could detect between 20 pg and 2.0 ng of 2,3,7,8-TCDD. The intra-assay precision of the [^{3}H] based assay was apparently better than that of the ^{125}I based assay's (<15% vs 20%). A "low end" version of the assay that used reduced levels of tracer (1500 cpm) and antiserum had a working range of 2.5-200 pg of 2,3,7,8-TCDD.

The modified assay is fast (overnight detection), sensitive (1.3 pg of 2,3,7,8-TCDD), and has a low detection limit (3.9 pg of 2,3,7,8-TCDD). The method is cost effective ($48 per sample, excluding sample extraction and cleanup; about $550 inclusive), easily learnt, and amenable to automation. RIA provides analysts with a tool that can be used to screen environmental samples for the presence of PCDDs. RIA can also be used to confirm the results of GC/MS analyses. The present report is intended to assist analysts who may wish to implement the method in their laboratories.

Scope and Application

The proposed method was tested using mono, di, tri, tetra, penta, hexa, hepta, and octachlorodibenzo-p-dioxin isomers. The method was also tested using three tetra-, two penta-, two hexa-, one hepta-, and one octachorodibenzofurans.

Using antiserum ALB-5, dose response relationships were established for several PCDD isomers other than 2,3,7,8-TCDD. The following PCDD isomers were observed to cross react significantly (≥20%): 2,7-Cl_2, 1,2,7,8-Cl_4, 1,3,7,8-Cl_4, 1,2,3,7,8-Cl_5, 1,2,3,4,7,8-Cl_6, 1,2,3,4,6,7,8-Cl_7. 1,2,3,4-TCDD cross reacted 19%. Octachloro-dibenzo-p-dioxin (DBD) appeared to cross react (21%); however in view of previous failures of

the RIA to detect Cl_8-DBD, that result is best considered provisional. DBD, 1-Cl, and 2-Cl did not cross react significantly. The following polychlorinated dibenzofuran (PCDF) isomers were observed to cross react significantly (≥20%): 2,3,6,8-Cl_4, 2,3,7,8-Cl_4, 1,2,7,8-Cl_4, 2,3,4,7,8-Cl_5, 2,3,4,6,8-Cl_5, 1,2,3,4,7,8-Cl_6, 2,3,4,6,7,8-Cl_6, 1,2,3,4,6,7,8-Cl_7, and octachloro-. The detection of these and other possible cross reacting PCDD and PCDF isomers is not considered to be a problem, since samples containing such compounds should also be analyzed using GC/MS techniques.

The RIA for PCDDs should be applicable to any environmental matrix, provided that suitable extraction and cleanup procedures are used. It is anticipated that most environmental matrices will need some cleanup before analysis by RIA. A streamlined extraction and clean-up method has been developed for this purpose (14).

Assay performance is dependent on the nature and size of the sample matrix and the degree of cleanup. Sample preparation will probably need to be matched to the matrix; research on this topic is in progress; with some modifications, the cleanup method that was used to prepare fish for analysis by the [^{125}I] based version of the RIA should suffice. In many cases, the RIA for PCDDs should require less cleanup than conventional GC/MS based methods.

RIA permits the simultaneous analysis of many sample replicates; an advantage that should be exploited to the full. Several hundred samples can be analyzed in a day; which could be particularly beneficial in the event of an environmental emergency.

Principle and Theory

Based on the classical antigen - antibody reaction (8), RIA is a relatively simple, powerful, and adaptable technique for the rapid determination of trace levels of organic compounds. Originally developed for the micro-determination of proteinaceous

substances (9), RIA is also extensively used to detect and determine steroidal hormones, antigens, drugs, and other low molecular weight organic molecules. According to The International Union Of Pure and Applied Chemistry, immunochemical methods, while being traditionally unfamiliar to the residue chemist, offer exciting possibilities for newer cost effective approaches (10).

In a typical RIA procedure antibodies and analyte, are pre-incubated together. Radio-ligand is then added to the assay tubes. The tubes' contents are mixed, and then re-incubated until equilibrium binding has occurred. At this stage one of several mechanisms is used to separate the bound from the free radioactivity. The bound (usually) radioactivity is quantified using a radiation counter, and a calibration curve is prepared. A reduction in the amount of bound radioactivity is proportional to the quantity of analyte present in the assay tube. Analyte concentration is interpolated from a standard curve.

Dioxin solubilization is a critical aspect of the assay. PCDDs are highly insoluble in aqueous solution, and yet binding with the water soluble antibodies must be facilitated. Dimethyl sulfoxide (DMSO) or non-ionic surfactants such as Triton X-305 or Cutscum can be used to aid solubilization.

After solubilization, the PCDD reference standards and cleaned-up sample extracts are incubated in their respective assay tubes together with the radio-ligand and antiserum; this allows competitive binding with the antibodies (ABs) to occur.

When equilibrium binding has been approached, the free radioactivity is rapidly adsorbed using Dextran coated charcoal. After centrifugation an aliquot of the supernatant, which contains the AB bound radioactivity, is quantified using a liquid scintillation counter.

An assay calibration curve is prepared by plotting the percentage of bound radioactivity, against the amount of 2,3,7,8-TCDD. The amount of PCDD in the sample extract is interpolated from the calibration curve. The amount of bound radioactivity in an assay tube is inversely proportional to the amount of PCDD.

A sample is considered positive if it is distinguishable from a matrix blank using Student's t test (95% confidence interval).

The assay precision profile (PP) helps establish the working range of the assay, and is determined by plotting the coefficient of variation (CV%) against amount of analyte. Although not an absolute value, the PP permits the meaningful evaluation of assay modifications, as well as providing a useful means of evaluating the performance of individual assays.

Assay sensitivity is defined as the precision of measurement of zero dose. It is estimated by plotting the standard deviation of the estimated analyte concentration against the actual amount of analyte present, and extrapolating the curve to zero dose. Both assay precision and sensitivity are determined by means of a replicated (10*) 10-14 point standard curve.

The assay detection limit is considered in two ways: the minimum detectable concentration (MDC) and the lowest concentration detected (LCD). The former is a deduced estimate of the DL based on statistical criteria (3 x the error associated with zero dose), whereas the latter is an empirical observation.

The slope of the dose response curve is a direct expression of the assay's responsiveness.

Interferences

The only known compounds capable of causing false positive results in the assay are dibenzo-p-dioxins substituted with halogens other than chlorine, and halogenated dibenzofurans. It may be possible to distinguish between these interferences using monoclonal antibodies. In any case, samples containing such interferences warrant further investigation. 3,4,3',4'-tetrachlorobiphenyl is known to weakly cross react with ALB-5 (6%) (2) and should not be a significant interference. Other co-planar PCBs have yet to be tested.

Residual hydrophobic matrix components that are capable of being solubilized by DMSO, or by either of the detergent systems can interfere with the solubilization of PCDDs or radio-ligand, by saturation of the solubilization agent.

Interference with PCDD solubilization can result in a reduced assay response or, in the extreme, a false negative result; a spiked sample will also give a false negative result in the latter case. Inclusion of a spiked matrix blank (matrix control), that has undergone the same preparation as the sample, will serve to alert the analyst, and in non-severe cases will permit the calculation of a correction factor. The solution to the problem lies in the careful selection of an appropriate cleanup procedure.

Interference with radio-ligand solubilization, can force the radioactive hapten from solution and cause an unusually elevated radiation count; this interference is apparent from the final tube counts. Inclusion of a carefully selected matrix blank, that has undergone the same preparation as the sample, will serve to alert the analyst, and in non-severe cases will permit the calculation of a correction factor. Again the careful selection of an appropriate cleanup procedure will solve the problem.

The presence of substances in the sample extract that can denature antibodies will cause false positive results. The use of matrix blanks will reveal the presence of such

interferences. Complete denaturation can be confirmed by the retesting of diluted sample. Matrix controls that yield much higher than expected TCDD levels are also indicative of AB denaturation. The assay incorporates several mechanisms to protect against such interferences e.g. EDTA and carrier proteins. The selection of a cleanup procedure that is carefully matched to the sample matrix will minimize this type of interference.

Matrix controls, which are matrix blanks that are spiked with 2,3,7,8-TCDD, should be included with each assay. Lower than expected responses from the matrix controls, in the absence of blank effects, is an indication that a hydrophobic sample component has solubilized the analyte and shielded it from the antibodies. In the absence of a matrix blank some of the sample replicates can be spiked in order to detect residual matrix interferences. This approach is less rigorous because it may fail to reveal low level false positive responses.

Spiked reagent blanks must be included with each set of samples in order to monitor assay instability or drift. At least 3 spiking levels should be used (low, medium, and high); these controls should be interspersed among the assay tubes.

Safety Precautions

Although the toxicity, particularly the chronic effects, of PCDDs to humans is incompletely understood (1), extreme care should be exercised when working with this class of compounds. 2,3,7,8-TCDD and several related isomers have proven, though variable, toxicity in mammalian test systems; most of the highly toxic isomers contain chlorine in the 2,3,7,8- positions. 2,3,7,8-TCDD is the most toxic man-made chemical yet tested in some test systems (eg guinea pig, rat, and mouse). Furthermore, 2,3,7,8-TCDD is a proven carcinogen in mammalian test systems (11), and thus may be carcinogenic to humans. The potential chronic health effects of dioxins to humans have

been largely extrapolated from data obtained using animal test systems. Skin contact, inhalation, or ingestion must be avoided.

Laboratories must be equipped with adequate exhaust systems and fume hoods.

Awareness and implementation of standard safety procedures and precautions will enable the analyst to safely work with this class of compounds.

All applicable federal or provincial regulations governing the use, transport, and disposal of radio-isotopes must be observed at all times.

All contaminated wastes must be stored in suitable containers for safe disposal.

Apparatus

Solvent Removal

- Solvent is removed from assay tubes by heat and vacuum assisted evaporation using a modified Buchler vortex evaporator with a capacity for six LKB tube racks. Vortex action was usually set to low: the precise setting will depend on the individual unit. The temperature of the evaporator was adjusted to 35°C. Solvent was removed from other tubes using a Pierce Reacti-Therm/Vap nitrogen blow down apparatus.

Radiation Counter

- LKB RackBeta 1217 liquid scintillation counter or equivalent. The counter is programmed to count light emissions caused by the [^{3}H] tracer, to automatically make the required quenching adjustments, and to list the results as DPM.

Centrifugation

- IEC DPR refrigerated centrifuge fitted with a rotor (IEC cat. # 227) designed to accommodate LKB tube racks.

Glassware

- Hamilton micro-syringes to cover the range 10-500 μL.
- glass tubes with teflon lined screw caps.
- glass beakers: 100 mL to 2 L.
- 12 mm * 75 mm disposable glass tubes with stoppers.
- pasteur pipettes.

In order to minimize the tendency of the assay tubes to adsorb PCDDs, all assay tubes should be pre-treated as follows: bring the tubes to the boil in a solution of 2% Micro in distilled water. After cooling, rinse the tubes 10 times with distilled water. Then boil the tubes in a solution of 1% Cutscum in distilled water. After cooling, rinse the tubes 10 times with distilled water and then once with pesticide grade acetone. Invert the tubes and allow them to dry in a fume hood.

Additional laboratory equipment

- 80°C freezer fitted with a CO_2 emergency back-up system.
- Constant temperature water bath that is fitted with a shaker motor.
- Low temperature (4°C) incubator.
- Ultrasonic water bath.
- Multi-tube vortex mixer.
- Single tube vortex mixer.
- Semi-automated pipetting station.
- Eppendorf adjustable pipettors.
- Positive displacement pipettors that accommodate glass tips.
- Membrane (0.2 μm) filtration apparatus for use in reagent sterilization.

  12 mm x 75 mm tube racks.

- LKB tube racks.
- Timer.
- Suction apparatus.
- Magnetic stirrer.

Reagents

Phosphate buffered saline (PBS):

A 500 mL stock solution of PBS contains 19 g of NaCl, 3.7 g of Na_2HPO_4 (anhydrous), 2.15 g of KH_2PO_4 (anhydrous), and 500 mg of NaN_3. The stock solution was adjusted to pH 7.3, filter sterilized and stored at 4°C. Dilutions (1 in 5) were made using de-ionised water; the pH of the diluted PBS was re-adjusted to 7.3.

Antibody diluent

Antibody diluent contained 100 mg of bovine gamma-globulin (Sigma G5009) per 100 mL of PBS. Antibody diluent should be prepared on the day of use.

Dextran coated charcoal reagent

1 g of dextran (Sigma D-1390) was allowed to swell in PBS (100 mL) overnight. 1 g of activated charcoal (Sigma C-5269) was then added, and the mixture was stirred for four hours. The DCC reagent is stored at 4°C. The amount of DCC reagent that must be added to the assay tubes in order to bind all the free tracer should be determined for each matrix type (6).

Antiserum to 3,7,8-TCDD

Antiserum was originally raised in rabbits against an antigen that had been prepared by conjugating 1-amino-3,7,8-trichlorodibenzo-p-dioxin (13) to bovine thyroglobulin by means of an adipamide linkage. The antiserum was lyophilized and

stored desiccated at 4°C. Reconstitute portions of the antiserum in distilled water and dilute (1:100 (v/v)) in antibody diluent. Store the diluted antiserum in 0.5 mL portions at -80°C.

Radio-ligand

Haptens are small molecules that are non-immunogenic unless conjugated to a carrier protein. The labelled hapten (radio-ligand) used in the present version of the RIA for 2PCDDs is 2,3,7,8-tetrachloro[1,6-^{3}H]dibenzo-p-dioxin (40 Ci/mmol) (Chemsyn Science Laboratories).

Other Reagents

Triton-X305 (Sigma).

Cutscum (Fisher C-547).

Dimethyl sulfoxide (Burdick and Jackson).

Deionized water - Milli-Q.

Toluene - distilled in glass (Burdick and Jackson).

Chloroform - distilled in glass.

Hexane - distilled in glass.

Acetone - distilled in glass.

2,3,7,8-TCDD reference standard (Cambridge Isotope Laboratories).

Preparation of radio-ligand solution

A stock solution of radio-ligand is prepared by dissolving [^{3}H]-2,3,7,8-TCDD (2 mCi) in toluene (2 mL); the stock solution is stored at -80°C. A 68 uL aliquot of the stock solution is added to a glass tube. The toluene is removed and replaced with 10 mL of DMSO. The radio-ligand solution is vortex mixed, ultrasonicated for 15 minutes, and then stored at 4°C.

Antiserum Standardization

Each batch of radio-ligand must be calibrated against the anti-dioxin serum using the following procedure:

- Label 30 assay tubes and arrange them in the LKB tube racks.
- Add 100 μL DMSO to each assay tube.
- Add 100 μL of PBS to each tube.
- Vortex mix the tubes.
- Add 200 μL of antibody diluent to the first three tubes: these are the BLANK tubes.
- Prepare a serial dilution of the antiserum in antibody diluent. The dilution series should be from 1:100 to 1:12000.
- Except for tubes #4-6 (the TOTAL tubes) each of the remaining triplicates receives 200 μL aliquots of the appropriately diluted antiserum, in ascending order.
- Stopper and then vortex mix the tubes.
- Incubate the tubes with shaking at 37°C for 40 minutes.
- Add 10000 cpm of radio-ligand in 50 μL of DMSO to each tube.
- Re-stopper the tubes and vortex mix.
- Incubate the tubes with shaking at 37°C for 60 minutes.
- Stand the tubes in a 4°C incubator overnight.
- On the following morning, precipitate the bound radioactivity using the DCC method (9.16).
- Count the precipitated radioactivity in each tube using a 6-10 minute count cycle.
- Subtract the counts from the blank triplicate from the other tubes. Plot the percentage of the radioactivity bound against antiserum dilution. Estimate the antiserum dilution required to bind 40 ± 5% of the added radioactivity.

Assay Protocol

- Label the assay tubes and arrange them in LKB tube racks. The standard curve should be located before the unknown tubes. The BLANK, TOTAL, and REFERENCE tubes are replicated 5 times. The 2,3,7,8-TCDD standards are replicated at least 3 and preferably 5 times. The unknowns, matrix blanks, matrix controls, and assay controls are all replicated the same number of times (preferably x5, but x3 will suffice). Each matrix blank is positioned near the unknown samples of its type, and the matrix controls are interspersed among the unknown samples. Low, medium, and high matrix controls (matrix blank containing a 2,3,7,8-TCDD spike) should be used; those controls should cover the assay's working range in (2,3,7,8-TCDD equivalents). Assay controls should be interspersed among the other tubes; these controls should be spiked at three levels: low = 50 pg, medium = 300 pg, and high =1000 pg.
- Add the appropriate quantities of 2,3,7,8-TCDD dissolved in DMSO to the standard tubes. The standard curve should cover the range 12.25 - 3200 pg 2,3,7,8-TCDD; the range can be varied to satisfy operational requirements.
- Add the unknowns and matrix blanks to the appropriate tubes. Spike the matrix controls and sample controls with 2,3,7,8-TCDD dissolved in DMSO and then vortex mix the tubes.
- Add 100 μL DMSO to the blank and reference tubes.
- Add 100 μL PBS to each tube and then vortex mix the tubes.
- Add 200 μL of standardized antiserum (8.16) to each tube, except the NSB and TOTAL tubes which receive AB diluent.
- Re-stopper the tubes and vortex mix.
- Incubate the tubes with shaking at 37°C for 40 minutes.

- Add 10000 cpm of radio-ligand in 50 μL of DMSO.
- Re-stopper the tubes and vortex mix.
- Incubate the tubes with shaking at 37°C for 60 minutes.
- Stand the tubes in a 4°C incubator overnight.
- On the following morning, precipitate the bound radioactivity as follows:
- All steps are performed at 4°C.
- Add 100 μL of DCC reagent to each tube with the exception of the TOTAL tubes which receive 100 uL of PBS.
- Vortex mix the tubes' contents.
- Incubate the tubes at 4°C for 5 minutes.
- Centrifuge the tubes at 3000 rpm for 45 minutes.
- Transfer 350 uL of each supernatant to a 20 mL vial that contains 10 mL of
- Scintiverse 11 (Fisher Scientific) scintillation cocktail.
- Count the vials in the liquid scintillation counter. The count times are adjusted so that at least 3000 counts are accumulated per assay tube.

Data Reduction and Analysis

- Determine the mean of each set of replicates.
- Subtract the BLANK reading from the other replicate sets.
- Calculate the percentage of radioactivity bound in the REFERENCE tubes. The assay is successful if 40 ± 5% of the TOTAL activity is bound.
- Plot (BOUND (B)/ REFERENCE (B_0)) x 100 versus Log_{10} (2,3,7,8-TCDD) for each standard.
- Unknowns are interpolated from the standard curve; they are expressed as 2,3,7,8-TCDD equivalents.

- Several assay trend parameters can be used to follow the performance of the RIA for PCDDs over a given period. The following parameters are recommended: REFERENCE/TOTAL, BLANK/REFERENCE, MDC, Slope, and ED20.

- For quality control purposes, assay controls and matrix controls can also be plotted. The drift within an assay can also be examined in this manner. Six controls can be located anywhere among the unknown samples. Controls can be repeated within an assay; the variation of the control from target value should be monitored.

Operational Notes and Considerations

Samples can be spiked with C^{13} -[1,2,3,4-TCDD] prior to extraction so as to monitor the recovery of TCDD; 1,2,3,4-TCDD is weakly bound by Alb 5 (approximately 16-20% cross reactivity). As other labelled tetra-TCDDs become available it may be possible to select a superior surrogate standard.

REFERENCES

NRCC, (1981). Polychlorinated dibenzo-p-dioxins: criteria for their effects on man and his environment. Associate Committee on Scientific Criteria for Environmental Quality. National Research Council of Canada. NRCC # 18574. 251 p..

Albro, P.W., M.I. Luster, K. Chae, S.K. Chaudhary, G. Clark, L.D. Lawson, J.T. Corbett and J.D. McKinney (1979) A radioimmunoassay for chlorinated dibenzo-p-dioxins. Toxicol. Appl. Pharmacol. 50: 137-146.

Afghan, B.K., J. Carron, P.D. Goulden, J. Lawrence, D. Leger, F. Onuska, J. Sherry and R.J. Wilkinson (1987) Recent advances in ultratrace analysis of dioxins and related halogenated hydrocarbons. Can. J. Chem. 65: 1086-1097.

Sherry, J.P., J.A. ApSimon, L. Collier, R.J. Wilkinson, P.W. Albro and B.K. Afghan 1989. The use of radioimmunoassay for the detection of polychlorinated dibenzo-p-dioxins in fish samples. Chemosphere 19: 255-261.

Sherry, J.P., J.W. ApSimon, T.L. Collier, and P.W. Albro, 1990. Use of dimethyl sulfoxide as solubilization agent in the detection of 2,3,7,8-TCDD by radioimmunoassay. Chemosphere 20: 1409-1416.

Sherry, J.P., and P.W. Albro, 1990. Radioimmunoassay of chlorinated dioxins: use of[^{3}H]-labelled 2,3,7,8-TCDD as radio-ligand. Presented at Dioxin '90, Bayreuth, Germany.

Sherry, J.P., J. ApSimon, L. Collier, B.K. Afghan, and P.W. Albro 1988. Radioimmunoassay for the detection of Polychlorinated Dibenzo-p-dioxins in Environmental Samples: Method Description. NWRI Contribution 88-73.

Atassi, M.Z., C.J. VanOss and D.R. Absolom (1984) Molecular Immunology. Marcel Dekker, New York. p. 725.

Yalow, R.S. and S.A. Berson (1960) Immunoassay of endogenous plasma insulin in man. J. Clin. Invest. 39: 1157-1175.

Hemingway, R.J., N. Aharonson, P.A. Greve, T.R. Roberts and H.P. Thier (1984) Improved cost-effective approaches to pesticide residues analysis. Pure & Appl. Chem. 56: 1131-1152.

Tschirley, F.H. (1986) Dioxin. Scientific American 254: 29-35.

Stanker, L.H., B. Watkins, N. Rodgers, and M. Vanderlaan, 1987. Monoclonal antibody for dioxin; antibody characterization and assay development. Toxicol. 45: 229-243.

Chae, K., L.K. Cho and J.D. McKinney (1977) Synthesis of 1-amino-3,7,8-trichlorodibenzo-p-dioxin and 1-amino-3,7,8-trichlorodibenzo-p-dioxin as haptenic compounds. Agric. Food Chem. 25: 1207-1209.

Sherry, J.P. and H. Tse, 1990. A procedure for the determination of PCDDs in fish. Chemosphere 20: 865-872.

APPENDIX 1. Monoclonal Antibody (MAB) version of RIA for the detection of PCDDs.

The MABs used in this version of the RIA were produced at Lawrence Livermore National Laboratories and have been described by Stanker et al. (12).

Antibody Standardization

Each batch of radio-ligand must be calibrated against the anti-dioxin MABs (DCC-3) using the following procedure:

- Label 30 assay tubes and arrange them in the LKB tube racks.
- Add 100 μL DMSO to each assay tube.
- Add 100 μL of PBS to each tube.
- Vortex mix the tubes.
- Add 200 μL of antibody diluent to the first three tubes: these are the BLANK tubes.
- Prepare a serial dilution of the MABs in antibody diluent. The dilution series should be from 15 to 2400 ng of MABs per RIA vial.
- Except for tubes #4-6 (the TOTAL tubes) each of the remaining triplicates receives 200 μL aliquots of the appropriately diluted antiserum, in ascending order.
- Stopper and then vortex mix the tubes.
- Incubate the tubes with shaking at 37°C for 40 minutes.
- Add 10000 cpm of radio-ligand in 50 μL of DMSO to each tube.
- Re-stopper the tubes and vortex mix.
- Incubate the tubes with shaking at 37°C for 60 minutes.
- Stand the tubes in a 4°C incubator overnight.

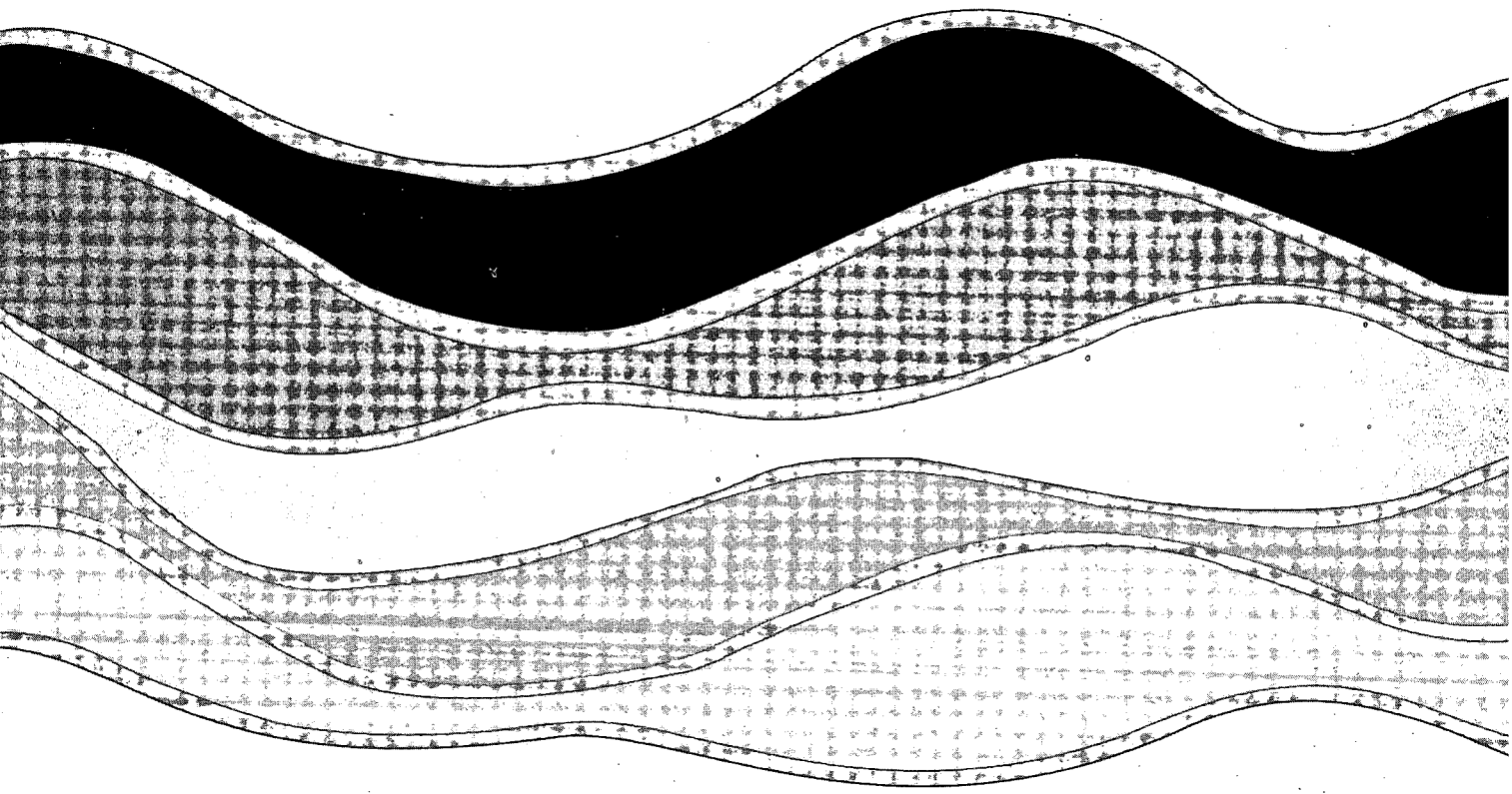
- On the following morning, precipitate the bound radioactivity using the DCC method (9.16).
- Count the precipitated radioactivity in each tube using a 6-10 minute count cycle.
- Subtract the counts from the blank triplicate from the other tubes. Plot the percentage of the radioactivity bound against quantity of MABs. Estimate the MAB dilution required to bind 40 ± 5% of the added radioactivity: it should be in the region of 90 - 100 ng.

Assay Protocol

- Label the assay tubes and arrange them in LKB tube racks. The standard curve should be located before the unknown tubes. The BLANK, TOTAL, and REFERENCE tubes are replicated 5 times. The 2,3,7,8-TCDD standards are replicated at least 3 and preferably 5 times. The unknowns, matrix blanks, matrix controls, and assay controls are all replicated the same number of times (preferably *5, but *3 will suffice). Each matrix blank is positioned near the unknown samples of its type, and the matrix controls are interspersed among the unknown samples. Low, medium, and high matrix controls (matrix blank containing a 2,3,7,8-TCDD spike) should be used; those controls should cover the assay's working range in (2,3,7,8-TCDD equivalents). Assay controls should be interspersed among the other tubes; these controls should be spiked at three levels: low = 50 pg, medium = 300 pg, and high = 1000 pg.
- Add the appropriate quantities of 2,3,7,8-TCDD dissolved in DMSO to the standard tubes. The standard curve should cover the

range 12.25 - 3200 pg 2,3,7,8-TCDD; the range can be varied to satisfy operational requirements.

- Add the unknowns and matrix blanks to the appropriate tubes. Spike the matrix controls and sample controls with 2,3,7,8-TCDD dissolved in DMSO and then vortex mix the tubes.
- Add 100 μL DMSO to the blank and reference tubes.
- Add 100 μL PBS to each tube and then vortex mix the tubes.
- Add 200 μL of standardized MABs (B.16) to each tube, except the NSB and TOTAL tubes which receive AB diluent.
- Re-stopper the tubes and vortex mix.
- Incubate the tubes with shaking at 37°C for 40 minutes.
- Add 10000 cpm of radio-ligand in 50 μL of DMSO.
- Re-stopper the tubes and vortex mix.
- Incubate the tubes with shaking at 37°C for 60 minutes.
- Stand the tubes in a 4°C incubator overnight.
- On the following morning, precipitate the bound radioactivity as follows:
- All steps are performed at 4°C.
- Add 100 μL of DCC reagent to each tube with the exception of the TOTAL tubes which receive 100 uL of PBS.
- Vortex mix the tubes.
- Incubate the tubes at 4°C for 5 minutes.
- Centrifuge the tubes at 3000 rpm for 45 minutes.
- Transfer 350 uL of each supernatant to 10 mL of Scintiverse 11 (Fisher Scientific) scintillation cocktail in a 20 mL vial.
- Count the vials in the liquid scintillation counter. The count times are adjusted so that at least 3000 counts are accumulated per assay tube.



NATIONAL WATER RESEARCH INSTITUTE
P.O. BOX 5050, BURLINGTON, ONTARIO L7R 4A6

Environment Canada Environnement Canada

Canada

INSTITUT NATIONAL DE RECHERCHE SUR LES EAUX
C.P. 5050, BURLINGTON (ONTARIO) L7R 4A6