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RADIOIMMUNOASSAY FOR THE DETECTION OF POLYCHLORINATED DIBENZO-P-DIOXINS IN ENVIRONMENTAL SAMPLES: METHOD DESCRIPTION

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MANAGEMENT PERSPECTIVE

The timely and cost effective analysis for 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, increase overall analytical efficiency. The radioimuunoassay (RIA) for PCDDs was developed to satisfy an IWD requirement for such a screening capability.

The interim version of the method described herein is a product of NWRI project 84-017 and has evolved during the course of evaluation, modification, and implementation studies. The sensitivity, detection limit, and speed of the DMSO based assay have proven superior to the detergent and horse serum based assays in evaluation studies using PCDD standards. The method is cost-effective ($48 per analysis, excluding sample preparation), rapid (overnight detection in the case of the DMSO based assay), easily learned, and amenable to automation. Further assay modifications are under evaluation.

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 large sample sets.

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PERSPECTIVE DE GESTION

L'analyse opportune et rentable pour le dépistage des polychlorodibenzoparadioxines (PCDD) est de la plus haute importance. Les méthodes conventionnelles pour la détermination des concentrations de PCDD dans des échantillons de l'environnement exigent beaucoup de temps et d'argent. L'emploi d'un test de dépistage permettra, grâce à l'élimination d'échantillons exempts de PCDD qui n'auront plus besoin d'être à nouveau analysés, d'augmenter l'efficacité globale de l'analyse. Le radioimmuno essai (RIA) pour la détection des PCDD a été mis au point pour répondre à une demande de la Direction générale des eaux intérieures.

La version provisoire de la méthode décrite ici est un produit du projet 84-017 de l'Institut national de recherche sur les eaux et elle a évolué au cours d'études d'évaluation, de modification et de mise en application. La sensibilité, la limite de détection et la rapidité de l'essai à base de CMSO se sont avérées supérieures à celles des essais à base de détergent et de sérum de cheval dans les études d'évaluation utilisant les normes des PCDD. La méthode est rentable (48 $ par analyse sans compter la préparation des échantillons), rapide (la détection se fait en une nuit dans le cas d'un essai à base de CMSO), facilement apprise et elle se prête à l'automatisation. D'autres modifications de l'essai sont entrain d'être évaluées.

Le RIA pour la détection des PCDD fournit aux analystes un outil pour dépister la présence de PCDD dans les échantillons de l'environnement. Le RIA peut également être utilisé pour confirmer rapidement les résultats des analyses GC/MS. Enfin cet essai devrait s'avérer utile dans les programmes de surveillance de l'environnement ainsi que dans l'analyse de grandes séries d'échantillons.

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ABSTRACT

The radioimmunoassay (RIA) for the detection of polychlorinated dibenzo-p-dioxins (PCDDs) is a screening test that can, by the elimination of PCDD free samples from further conventional analysis, improve analytical efficiency and lower the cost of determining dioxins in environmental samples. The present report describes a modified RIA procedure for the rapid detection of PCDDs in environmental samples. The assay is repeatable, rapid (overnight), sensitive (6 pg), and the least amount of 2,3,7,8-TCDD detectable in a lake trout sample was 10 pg. The detailed method description is intended as an aid for analysts wishing to implement the technique in a routine laboratory.

RÉSUMÉ

Le radioimmuno essai (RIA) pour la détection des polychlorodibenzoparadioxines (PCDD) est un test de dépistage préalable qui peut, en éliminant les échantillons exempts de PCDD pour qu'ils ne soient pas soumis à une autre analyse conventionnelle, améliorer l'efficacité de l'analyse et diminuer le coût du dosage des dioxines dans les échantillons de l'environnement. Ce rapport décrit une procédure modifiée du RIA qui permet de détecter rapidement les PCDD dans les échantillons de l'environnement. Cet essai peut être répété, il est rapide (en une nuit), sensible (6 pg), et la plus petite quantité de 2, 3, 7, 8-TCDD détectable dans un échantillon de truite fardée était de 10 pg. La description détaillée de la méthode vise à aider les analystes qui désirent appliquer la technique dans un laboratoire ordinaire.

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INTRODUCTION

Environmental persistence, exceptionally high toxicity, and accumulation in the food chain, have made the timely and cost effective analysis for the polychlorinated dibenzo-p-dioxins (PCDDs), and in particular, the notorious 2,3,7,8-TCDD isomer (NRCC, 1981) a high priority. However, conventional methods for the determination of PCDDs in environmental samples are time consuming and expensive - a single sample 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 as additional compounds are added to the already substantial list of agricultural and industrial chemicals that must be routinely monitored in the aquatic environment. An environmental catastrophe involving the release of PCDDs could create demands for the short term analysis of several hundred or thousand samples, including, in all probablility, human serum or biopsy samples; present analytical facilities would be unable to cope with such an exigency.

The inclusion of a screening test in the analytical protocol for the determination of PCDDs would help to solve the foregoing difficulty by eliminating those samples that are PCDD free from further time consuming and expensive conventional analysis. Immunoassay techniques are widely used in clinical laboratories for the detection and determination of antibodies, hormones, and medications in humans. Since many of these applications involve the detection of trace amounts of organic compounds, it seemed appropriate to consider the use of this technique for the detection of trace organic contaminants such as PCDDs in biotic and abiotic environmental matrices. The radioimmunoassay (RIA) for PCDDs was developed to satisfy an IWD requirement for such a screening capability with special reference to 2,3,7,8-TCDD and other PCDD isomers.

Originally developed by Albro et al. (1979), the radioimmunoassay (RIA) for the detection of PCDDs has been evaluated (Afghan et al., 1987) and modified in our laboratories. Various assay performance parameters such as precision, sensitivity, least amount detected, minimum detectable concentration, assay repeatability, potential interferences, and cross reacting compounds have been investigated. The assay has been interfaced with an extraction and cleanup procedure. The modified assay is fast (overnight detection), sensitive (6 pg of 2,3,7,8-TCDD), and has a low detection limit (15 pg of 2,3,7,8-TCDD). The method is cost effective ($48 per sample, excluding sample extraction and cleanup; $548 inclusive), rapid (overnight), easily learned, 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 rapidly 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. Further improvements to the method are being evaluated and, where beneficial, will be incorporated in later versions of the assay.

1.0 SCOPE AND APPLICATIONS

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

1.2 Using the available antiserum (ALB5) in the RIA, 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 ($\geq$20%); 2,7-Cl_2, 1,2,4-Cl_3, 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, DBD, 1-Cl, 2-Cl, and octachloro-DBD did not cross react significantly. 1,2,3-4-TCDD was observed to cross react 4% less than

the level (20%) reported by Alboro et al. (1979), but is being considered a significantly cross reacting isomer for now. The following PCDF isomers were observed to cross react significantly ($\geq$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. 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.

1.3 Fish tissues and river sediment have been analyzed. The technique should be applicable for any environmental matrix, provided that a suitable extraction and cleanup procedure are used. It is anticipated that most matrices will require cleanup before RIA analysis.

1.4 Fish matrix has been extensively evaluated. The minimum amount of sample cleanup necessary for a successful assay has been determined and the effect of sample size and degree of cleanup on some key assay performance characteristics has been investigated.

1.5 The extraction and cleanup procedures required will probably need to be varied depending on the matrix being analyzed; research on this topic is in progress. Assay performance is dependent on the nature and size of the sample matrix and the degree of cleanup; with some modifications, the cleanup method used for fish should suffice for most matrices. In most cases, it should be possible to reduce the amount of cleanup in comparison to that which is required for GC/MS without compromising assay performance. This aspect of the method is undergoing further research and development.

1.6 Simultaneous analysis of sample replicates is possible using RIA; this is an advantage that should be exploited to the full. Several hundred samples can be analyzed in a day, this could be particularly beneficial in the event of an environmental emergency.

2.0 PRINCIPLE AND THEORY

2.1 Based on the classical antigen-antibody reaction (Atassi et al., 1984), 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 (Yalow and Berson, 1960), RIA is also extensively used in the detection and determination of steroidal hormones, antigens, drugs and other low molecular weight organic molecules. Recently, the International Union of Pure and Applied Chemistry, reporting on improved cost effective approaches to pesticide residue analysis, concluded that immunochemical methods, while being traditionally unfamiliar to the residue chemist, offer exciting possibilities for newer cost effective approaches (Hemingway et al., 1984).

2.2 A typical RIA procedure is schematically represented in Figure 2. Antibodies and a sample containing analyte, are pre-incubated together. Labelled hapten 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 unbound radioactivity. The bound (usually) radioactivity is quantified using a gamma radiation counter, and a calibration curve is prepared. A reduction in the amount of radioactivity bound is theoretically proportional to the quantity of analyte present in the assay tube. Analyte concentration is interpolated from a standard curve.

2.3 The development of the radioimmunoassay for PCDDs required the preparation of two key reagents (Figure 1) - antibodies to the compound of interest, and a radio-labelled derivative of the compound of interest that could be bound by those antibodies (labelled hapten).

2.4 The antiserum used during method development (ALB5) was prepared in Dr. Albro's laboratory using the following procedure:

2.4.1 1-amino-3,7,8-trichlorodibenzo-p-dioxin was synthesized and converted to the adipamide derivative as described by Albro et al. (1979). The adipamide was converted to a mixed anhydride, which was then used to couple the dioxin derivative to bovine thyroglobulin, thus generating the required antigen. Particular care was taken to separate the protein bound moiety from free or adsorbed hapten: dialysis followed by extraction in chloroform was the technique of choice. The antigen was used to immunize New Zealand white rabbits. The resultant antiserum, which contained the antibodies to TCDD, was stored at -70°C until use.

2.5 Radioactive hapten is usually prepared by either labelling the compound of interest directly with ^{125}I, or if the iodine interferes with the compounds immuno-reactivity, a suitable side chain can be used to separate the tracer from the main body of the molecule. The labelled hapten used in the RIA for PCDDs is prepared using the following proecdure:

2.6 1-amino-3,7,8-trichlorodibenzo-p-dioxin is converted to 1-N-(5-iodovaleramido)-3,7,8-trichlorodibenzo-p-dioxin. The product is separated from free ^{125}I by means of silica gel chromatography. ^{125}I is a gamma radiation emitting radioisotope with a half life of two months; its radioactive output can be quantified using commercially available monitors.

2.7 Because of the half life of ^{125}I, fresh batches of labelled hapten should be prepared every two months. The antiserum must be re-standardized against each batch of labelled hapten, so as to determine the antiserum dilution required to bind 40±5% of the radioactivity. During the working life of the labelled hapten, the dilution of antiserum used in the assay should be continually adjusted to compensate for the decay of the radiotracer. Radical departures from the desired degree of binding are indicative of

reagent problems, e.g., hapten deterioration, incorrect dilutions, etc., and should be investigated and corrected.

2.8 Dioxin solubilization is a critical aspect of the assay. PCDDs are highly insoluble in aqueous solution, and yet binding with water soluble antibodies must be facilitated, if the assay is to be successful. The original assay used detergent (Cutscum or Triton X-305) micelles to solubilize the highly insoluble PCDD molecules. Dimethyl sulfoxide (DMSO) offers several advantages over the detergent based solubilization procedures - improved rapidity, ease of solubilization, and improved assay performance parameters.

2.9 After solubilization, the PCDD reference standards and cleaned up sample extracts are incubated in their respective assay tubes together with the labelled hapten and antiserum, thus facilitating competitive binding between the antibodies, the labelled hapten, and PCDD.

2.10 The RIA for PCDDS is an accelerated double antibody procedure. When equilibrium binding has been reached, bound and free radioactivity are separated by the addition of an antibody to rabbit gamma globulin, the resultant precipitate removes the antibody bound radioactivity from solution, some carrier rabbit gamma globulin is included in the assay medium to assist in this process which is accelerated by the use of polyethylene glycol. After centrifugation and aspiration of the supernatant, the bound radioactivity is subsequently quantified using a gamma radiation counter interfaced to an Olivetti M-24 computer.

2.11 An assay calibration curve is prepared by plotting bound radioactivity, or a function thereof, against 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. Counter control, data logging and reduction, curve fitting, interpolation of standards, and quality control are all executed by the

computer software. User over-ride of the computer is possible at all times.

2.12 A sample is considered positive if it is distinguishable from a matrix blank using Student's t test.

2.13 The assay precision profile 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, when determined using environmental samples, the precision profile permits the meaningful evaluation of assay modifications, as well as providing a useful means of evaluating the performance of individual assays.

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

2.15 Assay detection limit is defined in two ways - minimum detection concentration (MDC) and lowest concentration detected (LCD). The former is a deduced estimate of the lowest concentration of analyte that can be distinguished from zero using statistical criteria, whereas the latter is an empirical observation.

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

3.0 INTERFERENCES

3.1 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 which are now becoming available. In any case, samples containing such interferences warrant further

investigation using GC/MS. 3,4,3,4-tetrachlorobiphenyl is known to cross react with the antibodies to TCDD (Albro et al., 1979), but only at a level of 6% cross reactivity; this should not be a significant interference.

3.2 Residual hydrophobic matrix components capable of being solubilized by DMSO, or by either the Triton or horse serum solubilization agents used in the alternative versions of the assay (Appendices 1 and 2), can interfere with the solubilization of (1) PCDDs or (2) labelled hapten, by saturation of the solubilization agent.

3.2.1 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 carefully selected matrix blank, that has undergone the same preparation as the sample, and is spiked with TCDD (matrix control), 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.

3.2.2 Interference with labelled hapten solubilization, can force the radioactive hapten from solution and results in the detection of an unusually elevated radiation count; this phenomenon is immediately 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. The solution to the problem lies in the careful selection of an appropriate cleanup procedure.

3.3 The presence of substances in the cleaned-up 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 dilution of the sample and re-testing. Matrix controls that yield higher

than expected TCDD levels are also indicative of such problems. The assay incorporates several mechanisms to protect against such interferences, e.g., EDTA and carrier proteins. The selection of an appropriate cleanup procedure that is carefully matched to the sample matrix will minimize this type of interference.

3.4 Matrix controls, which are matrix blanks spiked with several levels of 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 TCDD and is shielding 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 that can result from positive matrix interferences.

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

4.0 SAFETY PRECAUTIONS

4.1 Although the toxicity, particularly the chronic effects, of PCDDs to humans is not completely understood (NRCC, 1981), it is clear that extreme care should be exercised when working with the class of compounds, because of the proven, though variable, toxicity of 2,3,7,8-TCDD and several related isomers in mammalian test systems. Most of the highly toxic isomers also contain chlorine in the 2,3,7,8- positions. 2,3,7,8-TCDD is thought to be the most toxic man-made chemical yet tested in some of these systems (e.g., guinea pig, rat, and mouse). Furthermore, 2,3,7,8-TCDD is a proven carcinogen in mammalian test systems (Tschirley, 1986) and thus may have carcinogenic potential toward 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 at all costs.

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

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

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

4.4.1 Labelling of the hapten with ^{125}I must be carried out in a medium level radio-isotope laboratory. The operator must be familiar with the safety procedures used in such work, e.g., the use of lead-shielding, iodide stabilization solutions, and triple layers of safety gloves. The radio-labelled hapten should be stored in screw-capped glass tubes, which are inserted into beakers of lead shot, thus reducing their radioactive field.

4.4.2 Personnel involved in labelling reactions must have their thyroid glands monitored for radioactive contaminations, preferably on the day after the labelling reactions has been completed. Operators should routinely monitor their hands, bench surfaces, and the general work area for accidental contamination.

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

5.0 APPARATUS

5.1 Solvent Removal: Solvent was removed from assay tubes using a modified Buchler vortex evaporator that accommodated six LKB tube racks, each with a capacity of 10 tubes. Vortex action was

usually set to low; the precise setting will depend on the individual unit. Temperature was adjusted to 35°C for use with benzene, toluene, or methanol.

5.2 Radiation Counter: LKB 1271 RIAGAMMA radiation counter, or equivalent, which detects ^{125}I with an efficiency of 75% or better. The counter is controlled by an Olivetti M-24 computer using the RiaCalc LM software package (LKB).

5.3 Centrifugation: IEC DPR refrigerated centrifuge fitted with a rotor designed to accommodate LKB tube racks.

5.4 Glassware:

- 250 and 500 mL Erlenmeyer flasks.
- Allihn filters with 4 cm x 10 cm reservoir and sintered glass disks of grade 8 porosity.
- Hamilton micro-syringes to cover the range 10-500 L.
- Chromatographic column 30 cm x 1 cm fitted with teflon stopcock and a removable glass reservoir.
- Various sizes of glass beaker from 100 mL to 2 L.
- 12 mm x 75 mm disposable glass tubes with stoppers.

In order to minimize binding of PCDDs to glass, all assay tubes must be pretreated in the following manner. Bring the tubes to the boil in a solution of 2% Micro in distilled water. After cooling, rinse the tubes ten 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. The tubes are then inverted and dried in a fume hood.

- Pasteur pipettes.

5.5 Additional laboratory equipment:

5.5.1 -80°C freezer fitted with a CO_2 emergency backup system.

5.5.2 Constant temperature shaker water bath.

5.5.3 Ultrasonic water bath.

5.5.4 Multi-tube vortex mixer.

5.5.5 Single tube vortex mixer.

5.5.7 Semi-automated pipetting station.

5.5.8 Eppendorf adjustable pipettors.

5.5.9 SMI adjustable positive displacement pipettors.

5.5.10 Membrane (0.2 μm) filtration apparatus for use in reagent sterilization.

5.5.11 Basin for holding crushed ice.

5.5.12 RIA tube racks.

5.5.13 LKB tube racks.

5.5.14 Timer.

5.5.15 Suction apparatus connected to an aspirator.

5.5.16 Magnetic stirrer.

5.5.17 Hotplate.

5.5.18 Thermostatically controlled block heater or sand bath that can accommodate the lead pig used for shipping ^{125}I.

5.5.19 Thermometer.

5.5.20 Lead shot.

6.0 REAGENTS

6.1 Phosphate Buffered Saline (PBS): A 500 mL stock solution of PBS contained 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-ionized water.

6.2 Antibody Diluent: Antibody diluent contained 100 mg of bovine gamma-globulin (Sigma G5009) and 20 mg of rabbit gamma-globulin (Sigma G0261) per 100 mL of PBS. Antibody diluent should be prepared on the day of use.

6.3 Second Antibody Reagent: Twenty mL of PBS containing 0.05 mM ethylenediaminetetraacetic acid disodium salt (EDTA) are added to a vial of goat anti-rabbit gamma-globulin (Calbiochem-Behring Corp. 539844; 125 units). A 200 μL aliquot of this reagent is added to each assay tube.

6.4 Horse Serum Reagent: 10% (w/v) solution of horse serum (Sigma S6380) dissolved in PBS and then filter sterilized.

6.5 Antiserum to 3,7,8-TCDD: Antiserum was raised in rabbits against an antigen that had been prepared by conjugating 1-amino-3,7,8-trichlorodibenzo-p-dioxin to bovine thyroglobulin by means of an adipamide linkage. The antiserum was lyophilized and stored dessicated at 4°C. Reconstitute portions of the antiserum in distilled water and dilute 1:50 (v/v) or 1:100 (v/v) in antibody diluent. Store the diluted antiserum in 0.5 mL portions at -80°C until use.

6.6 Hapten: Synthesize the unlabelled hapten, 1-N-(5-iodovaleramido)-3,7,8-trichlorodibenzo-p-dioxin as described by Albro et al. (1979) and Chae et al. (1977). GC analysis can be used to confirm the presence of a single compound. Electron impact mass spectroscopy should indicate the correct mass (less HI) for the hapten. The hapten may be further characterized, or checked before use, using thin layer chromatography on pre-coated silica gel TLC plates (Sybron Brinkman, SilG-UV254) using benzene as the solvent; after solvent removal, the plates are examined under UV light (254 nm).

The following procedure is used for the routine storage and manipulation of the hapten:

- Store the dry hapten under nitrogen at -80°C.
- Equilibrate the hapten to room temperature in a desiccator, before dissolving in $CHCl_3$.
- Remove a portion of the hapten solution (approximately 10 mL) and add to a solvent rinsed glass beaker. Add dry acetone (90 mL) to the beaker and mix. Remove the solvent under a stream of nitrogen until approximately 20 mL remains in the beaker. Again add dry acetone (50 mL) to the beaker and mix; repeat the solvent removal until about 6 mL of solvent remains.

- Transfer the hapten solution to a volumetric flask and adjust to 10 mL.
- Ultrasonication is used throughout the foregoing procedure to assist solubilization.

6.7 Other Reagents:

- Triton-X305
- Cutscum
- Dimethyl sulfoxide
- Deionized water - Millipore
- Toluene - distilled in glass
- Chloroform - distilled in glass
- Hexane - distilled in glass
- Acetone - distilled in glass
- Sodium Sulphate, heated at 650°C for 24 hours and stored desiccated
- 2,3,7 8-TCDD reference standard
- Iodide stabilization solution: consists of 0.1 M NaI, 0.1 M NaOH, and 0.1 M $Na_2S_2O_3$ in deionized H_2O
- Polyethylene Glycol Reagent: 4 g of polyethylene gylcol (Sigma P2139, MW 8000) dissolved in 100 mL PBS.

7.0 PREPARATION OF LABELLED HAPTEN

The hapten is rendered radioactive by exchanging its iodo group for ^{125}I using the following procedure:

7.1 Dissolve the unlabelled hapten in dry acetone using the following procedure:

7.1.1 Remove the dry hapten from the -80°C deep freeze, and allow to equilibrate with room temperature. Dissolve in 10 mL of $CHCL_3$. Remove about 400 g of hapten and transfer to a clean solvent rinsed beaker, add 90 mL of dry acetone and mix the solvents. Evaporate off the solvents under a gentle stream of nitrogen

until about 20 mL remains. Add 50 mL of dry acetone to the beaker and evaporate down to about 7 mL in the same manner. Transfer to a 10 mL volumetric flask and adjust to the mark.

7.2 Inject 10 µg of hapten in approximately 250 µL of dry acetone into a vial that contains 5mCi of previously centrifuged (400 rpm for 5 minutes) $Na^{125}I$ (Amersham, IMS300, carrier-free). The hapten solution is injected through the septum in the centre of the vial's cap.

7.3 Incubate the vial and its contents at 50°C for 66 hr in a thermostatically controlled heating block.

7.4 Extract and purify the reaction products using the following procedure:

7.4.1 Allow the reaction mixture to equilibrate with room temperature.

7.4.2 Transfer the reactants to a screw-capped glass tube that contains 10 mL of chloroform. Rinse the tracer vial with dry acteone, and add to the remainder of the reaction products.

7.4.3 Thoroughly mix the tube contents for one minute.

7.4.4 Add 2 mL of 10% (w/v) sodium metabisulphite to the tube, and again thoroughly mix the contents.

7 4.5 Remove and discard the aqueous layer, which contains free ^{125}I.

7.4.6 Add 2 mL of dionized H_2O to the tube, and again thoroughly mix the contents for one minute.

7.4.7 Remove and discard the aqueous layer.

7.4.8 Dry the extract by passing through anhydrous sodium sulphate.

7.4.9 Remove the chloroform under stream of nitrogen.

7.4.10 Redissolve the reaction product(s) in hexane and apply to a silica gel column (10 mm x 170 mm; 60-200 mesh). Elute the column with hexane to remove residual free ^{125}I, and then with $CHCl_3$:hexane 1:1 to elute the organic reaction product(s). Collect 10 mL fractions in solvent rinsed glass tubes that are fitted with teflon lined caps; store at -80°C.

The labelled hapten is characterized using both TLC and immunoassay procedures (RIA scan). Thin layer chromatography is carried out as previously described for the unlabelled hapten (6.6). The developed plates are scanned for gamma radiation using a Packard TLC plate scanner, or equivalent.

7.5 RIA Scan: Radioactive fractions from 7.4 must be characterized using the following procedure:

7.5.1 Bring the radioactive fractions from 7.4.10 to room temperature. Count 20 L aliquots. Calculate the total amount of radioactivity in each tube. Add 2 x 10^6 cpm of radioactivity from each tube to a solvent rinsed screw-capped glass tube. Remove the solvent under a gentle stream of nitrogen. Add 5 mL DMSO to each tube; vortex mix, and then ultrasonicate for 15 minutes. Count 20 L aliquots from each tube and determine the blow-down loss, if any.

7.5.2 Arrange and label three triplicates of assay tubes per fraction in LKB tube racks.

7.5.3 Add 100 µL of DMSO to each tube.

7.5.4 Add 100 µL of PBS to each tube.

7.5.5 Vortex mix the tubes.

7.5.6 Add 200 µL of antibody diluent to the first triplicate of each fraction, this is the BLANK triplicate.

7.5.7 Add 200 µL of antiserum diluted 1:1000 in antibody diluent to the second (REFERENCE) triplicate for each fraction.

7.5.8 Stopper the tubes and then vortex mix.

7.5.9 Incubate the tubes at 37°C for 40 minutes.

7.5.10 For each fraction, add 7000 CPM of tracer in 50 µL of DMSO to the corresponding nine tubes. The third triplicate is the TOTAL activity reference for each fraction.

7.5.11 Re-stopper the tubes and vortex mix.

7.5.12 Incubate the tubes with shaking at 37°C for 60 minutes.

7.5.13 Then, incubate the tubes at 4°C overnight.

7.5.14 On the following morning, precipitate the bound radioactivity using the accelerated procedure (9.16).

7.5.15 Count the precipitated radioactivity in each tube using a 10 minute count cycle.

7.5.16 Subtract the counts of the blank triplicate from the other tubes.

7.5.17 Calculate the percentage of radioactivity bound for each fraction.

7.5.18 The fraction with the highest binding level is used in the RIA.

7.6 Store the selected fraction (7.6.19) in 0.5 mL aliquots at -80°C.

8.0 ANTISERUM STANDARDIZATION

Each batch of labelled hapten must be calibrated against the antidioxin antiserum using the following procedure:

8.1 Label 30 assay tubes and arrange them in LKB racks.

8.2 Add 100 µL DMSO to each assay tube.

8.3 Add 100 µL of PBS to each tube.

8.4 Vortex mix the tubes.

8.5 Add 200 µL of antibody diluent to the first three tubes: these are the BLANK tubes.

8.6 Prepare a serial dilution of the anti-dioxin antiserum using antibody diluent. The dilution series should run from 1:1000 to 1:128000.

8.7 Except for tubes #4-6 (the TOTAL tubes) each of the remaining triplicates receives 200 µL aliquotes of the appropriately diluted antiserum, in ascending order.

8.8 Stopper the tubes and vortex mix.

8.9 Incubate the tubes with shaking at 37°C for 40 minutes.

8.10 Add 7000 cpm of labelled hapten in 50 µL of DMSO to each tube.

8.11 Re-stopper the tubes and vortex mix.

8.12 Incubate the tubes with shaking at 37°C for 60 minutes.

8.13 Transfer the tubes to a 4°C incubator and incubate overnight.

8.14 On the following morning, precipitate the bound radioactivity using the accelerated procedure (9.16).

8.15 Count the precipitated radioactivity in each tube using a 10 minute count cycle.

8.16 Subtract the counts from the blank triplicate from the other tubes. Using RIACALC plot the percentage of the radioactivity bound against antiserum dilution. Interpolate the antiserum dilution required to bind 40∓5% of the added radioactivity.

8.17 This dilution should be adjusted to compensate for radio-tracer decay during the 60 day shelf life of the labelled hapten.

9.0 ASSAY PROTOCOL

9.1 Preparation of Tracer Solution.

9.2 Add 2 x 10^6 cpm of labelled hapten to a screw-capped glass tube.

9.3 Remove the solvent under a gentle stream of nitrogen.

9.4 Add 5 mL of DMSO reagent to the tube.

9.5 Vortex mix for 20 seconds every minute over a 15 minute period.

9.6 Ultrasonicate the tubed contents for 30 minutes.

9.7 Count 10 µL, and calculate the volume of solution that contains 7000 cpm.

9.8 Store at 4°C in aliquots.

9.9 Label the required number of assay tubes and arrange them in LKB tube racks. Tubes for the standard curve are located before the unknown tubes. The BLANK, TOTA, and REFERENCE tubes are replicated five times. The 2,3,7,8-TCDD standards are replicated at least three and preferably five times. The unknowns, matrix blanks, matrix controls, and assay controls are all replicated the same number of times (preferably x5, but x3 will suffice). The matrix blank is located with the unknown samples of its type. The matrix controls are interspersed among the other tubes, and should be spiked at three levels: low = 50 pg, medium = 30 pg, and high = 1000 pg. RIACALC should be informed of the locations of all tube types.

9.10 Add the appropriate quantities of 2,3,7,8-TCDD dissolved in toluene to the standard tubes. The standard curve should cover the range 12.25 - 2000 pg 2,3,7,8-TCDD, although the range can be varied to satisfy operational requirements.

9.11 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 toluene and vortex mix.

9.12 Evaporate off the solvent in the Buchler evaporator at 37°C and 30 atm. for 30 minutes.

9.13 Add 200 µL of toluene to each tube; vortex mix, to rinse the tube walls, and then evaporate off the solvent as previously described (3.4).

9.14 Add 100 µL DMSO to each tube.

9.15 Stopper the tubes and vortex mix.

9.16 Ultrasonicate for 15 minutes.

9.17 Add 10 µL PBS and vortex mix.

9.18 Add 200 µL of standardized antiserum (8.16) to each tube, except the TOTAL tubes.

9.19 Re-stopper the tubes and vortex mix.

9.20 Incubate the tubes with shaking at 37°C for 40 minutes.

9.21 Add 7000 cpm of labelled hapten in 50 µL of DMSO.

9.22 Re-stopper the tubes and vortex mix.

9.23 Incubate the tubes with shaking at 37°C for 60 minutes.

9.24 Transfer the tubes to a 4°C incubator and incubate overnight.

9.25 On the following morning, precipitate the bound radioactivity using the following accelerated procedure:

9.25.1 All steps are executed at 4°C.

9.25.2 Add 200 µL of second antibody reagent to each tube with the exception of the TOTAL tubes.

9.25.3 Re-stopper the tubes and vortex mix.

9.25.4 Incubate the tubes at 4°C for two hours.

9.25.5 Add 1 mL of PEG reagent to each tube with the exception of the TOTAL tubes.

9.25.6 Re-stopper the tubes and vortex mix.

9.25.7 Centrifuge the tubes at 2000 rpm for 30 minutes.

9.25.8 Aspirate the supernatant from each tube except the TOTAL tubes.

9.25.9 Drain the tubes onto absorbent paper for 10 minutes.

9.25.10 Wipe the inside and lip of each tube using a twirled KIMWIPE^{tm}.

9.26 Restopper the tubes and count in the gamma counter using a 10 minute count cycle.

10.0 DATA REDUCTION AND ANALYSIS

10.1 All data reduction and analysis can be done by means of the RIACALC software package. The following is a description of one of several calculation methods that can be used, the others are described in the RIACALC manual.

10.2 Determine the mean of each set of replicates.

10.3 Subtract the BLANK reading from all other replicate sets.

10.4 Calculate the percentage of radioactivity bound in the REFERENCE tubes. The assay is successful if 40$\mp$5% of the TOTAL activity is bound.

10.5 Plot BOUND (B)/REFERENCE (B_0) versus Log (2,3,7,8-TCDD) for each standard. Use the third order spline routine in RIACALC to plot and automatically smooth a line through the standard points. Outlier points can be eliminated at this stage, visual criteria, or a statistical outlier test can be used. Store the standard curve for comparison with future assays.

10.6 Unknowns are interpolated from the standard curve, they are expressed as 2,3,7,8-TCDD equivalents.

10.7 Use RIACALC to plot and store a precision profile for the assay, store the precision profile for comparison with future assays.

10.8 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; they can be automatically plotted and stored on disk by RIACALC for retrieval at the analysts convenience.

10.9 For quality control purposes, assay controls and matrix controls can also be plotted and stored on disk. The drift within an assay can also be examined in this manner. Six controls can be managed, and they 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.

11.0 OPERATIONAL NOTES AND CONSIDERATIONS

11.1 Samples can be spiked with C^{13} labelled 1,2,3,4-TCDD prior to extraction so as to monitor the recovery of TCDD from the sample, 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.

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APPENDIX A

TRITON VERSION OF ASSAY PROTOCOL

1.0 The following protocol can be used for other detergent-based versions of the assay (e.g., the Cutscum version).

2.0 Preparation of Tracer Solution:

2.1 Add 2 x 10^6 cpm of labelled hapten to a screw-capped glass tube.
2.2 Remove the solvent under a gentle stream of nitrogen.
2.3 Add 150 µL of a 2:1 solution of methanol and Triton X-305 (v/w), and thoroughly mix the tube's contents.
2.4 Remove the methanol under a gentle stream of nitrogen.
2.5 Add 4.45 mL of PBS and vortex mix the tube's contents.
2.6 Ultrasonicate the tubes for 40 minutes.
2.7 Centrifuge at 500 rpm for 30 minutes, and transfer 4.4 mL of the supernatant to a clean glass tube. Count 10 µL, and calculate thevolume of solution that contains 700 cpm.
2.8 Store at 4°C; the hapten should remain stable under these conditions for 2-3 weeks.

3.0 Antiserum Standardization:

3.1 Each batch of labelled hapten must be calibrated against the anti-dioxin antiserum using the following procedure:
3.2 Label 24 assay tubes and arrange them in LKB racks.
3.3 Add 200 µL of 1% (w/v) Triton in methanol to each assay tube.
3.4 Vortex mix the tubes.
3.5 Evaporate off the methanol using the buchler evaporator at 35°C and 30 atm. for 30 minutes.
3.6 Add 200 µL of PBS to each tube.
3.7 Stopper the tubes and then vortex mix.
3.8 Ultrasonicate the tubes for 40 minutes.

3.9 Add 200 µL of antibody diluent to the first three tubes, these are the BLANK tubes.

3.10 Prepare a serial dilution of the anti-dioxin antiserum using antibody diluent. The dilution series should run from 1:1000 to 1:32000.

3.11 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.

3.12 Re-stopper the tubes and vortex mix.

3.13 Incubate the tubes with shaking at 37°C for 40 minutes.

3.14 Add 7000 cpm of labelled hapten in 50 µL of Triton/PBS solution to each tube.

3.15 Re-stopper the tubes and vortex mix.

3.16 Incubate the tubes with shaking at 37°C for 60 minutes.

3.17 Transfer the tubes to a 4°C incubator and incubate for 68-72 hours.

3.18 Precipitate the bound radioactivity using the accelerated procedure (9.16).

3.19 Count the precipitated radioactivity in each tube using a 10 minute count cycle.

3.20 Subtract the counts from the blank triplicate from the other tubes. Using RIACALC plot the percentage of the added radioactivity bound against antiserum dilution. Interpolate the antiserum dilution required to bind 40 5% of the added radioactivity.

3.21 This dilution should be adjusted to compensate for radiotracer decay during the 60 day shelf life of the labelled hapten.

4.0 Assay Protocol

4.1 Arrange the assay tubes as described in 9.1.

4.2 Add the appropriate quantity of 2,3,7,8-TCDD standard in toluene to the standard tubes. The standard curve should cover the range 25-4000 pg, although the range can be adjusted to satisfy operational requirements.

4.3 Add the unknowns and matrix blanks, which are prepared in toluene, to the appropriate tubes. Spike the matrix controls and sample controls with 2,3,7,8-TCDD in toluene. Vortex mix the tubes.

4.4 Evaporate off the solvent using the Buchler evaporator at 37°C and 30 atmosphere for 30 minutes.

4.5 Add 200 µL of toluene to each tube; vortex mix, to rinse the tube walls, and then evaporate off the solvent as previously described (3.4).

4.6 Add 200 L of 1% (w/v) Triton dissolved in methanol to each tube; cover the tubes with aluminum foil.

4.7 Vortex mix to 20 seconds every minute over a 5 minute period.

4.8 Evaporate off the methanol as previously described (3.4).

4.9 Add 0.2 mL of PBS to each tube.

4.10 Stopper the tubes and then vortex mix.

4.11 Ultrasonicate the tubes for 40 minutes; the starting temperature of the bath should be adjusted to 4°C.

4.12 Vortex mix the tubes twice.

4.13 Cover the tubes in aluminum foil and stand overnight at 4°C. This step is optional, but convenient for a lone operator.

4.14 Allow the tubes to equilibrate with room temperature.

4.15 Add 200 µL of antibody diluent to the BLANK tubes.

4.16 Add 200 µL of standardized antiserum (8.16) to each of the remaining tubes, except the TOTAL tubes.

4.17 Re-stopper the tubes and vortex mix.

4.18 Incubate the tubes with shaking at 37°C for 40 minutes.

4.19 Add 7000 cpm of labelled hapten to each tube.

4.20 Re-stopper the tubes and vortex mix.

4.21 Incubate the tubes with shaking at 37°C for 60 minutes.

4.22 Transfer the tubes to a 4°C incubator and incubate for 68-72 hours.

5.0 Precipitate the bound radioactivity using the accelerated procedure (9.16).

5.1 Aspirate the supernatant from each tube except the TOTAL tubes.

5.2 Drain the tubes onto absorbent paper for 10 minutes.

5.3 Wipe the inside and lip of each tube using a twirled KIMWIPE^{tm}.

5.4 Re-stopper the tubes and count in the gamma counter using a 10 minute count cycle.

APPENDIX B

HORSE SERUM VERSION OF ASSAY PROTOCOCOL

1.0 Preparation of Tracer Solution.

1.1 Add 2 x 10^6 cpm of labelled hapten to a screw-capped glass tube.
1.2 Remove the solvent under a gently stream of nitrogen.
1.3 Add 5 mL of horse serum reagent to the tube.
1.4 Vortex mix for 20 seconds every minute over a 15 minute period.
1.5 Ultrasonicate the tubes for 30 minutes.
1.6 Count 10 µL, and calculate the volume of solution that contains 7000 cpm.

2.0 Antiserum Standardization

2.1 Each batch of labelled hapten must be calibrated against the anti-dioxin antiserum using the following procedure:
2.2 Label 24 assay tubes and arrange them in LKB racks.
2.3 Add 200 µL of horse serum reagent to each tube.
2.4 Add 200 µL of antibody diluent to the first three tubes; these are the BLANK tubes.
2.5 Prepare a serial dilution of the anti-dioxin antiserum using antibody diluent. The dilution series should run from 1:1000 to 1:32000.
2.6 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.
2.7 Re-stopper the tubes and vortex mix.
2.8 Incubate the tubes with shaking at 37°C for 40 minutes.
2.9 Add 7000 cpm of labelled hapten.

2.10 Re-stopper the tubes and vortex mix.

2.11 Incubate the tubes with shaking at 37°C for 60 minutes.

2.12 Transfer the tubes to a 4°C incubator and incubate for 68-72 hours.

2.13 Precipitate the bound radioactivity using the accelerated procedure (9.16).

2.14 Count the precipitated radioactivity in each tube using a 10 minute count cycle.

2.15 Subtract the BLANK counts from those of the other tubes.

2.16 This dilution should be adjusted to compensate for radiotracer decay during the 60 day shelf life of the labelled hapten.

3.0 Assay Protocol

3.1 Arrange the assay tubes as described in 9.1.

3.2 Add the appropriate quantity of 2,3,7,8-TCDD standard dissolved in toluene to the standard tubes. The standard curve should cover the range 25 - 16000 pg 2,3,7,8-TCDD, although the range can be varied to satisfy operational requirements.

3.3 Add the unknowns and matrix blanks, which are prepared in toluene, to the appropriate tubes. Spike the matrix controls and sample controls with 2,3,7,8-TCDD dissolved in toluene. Vortex mix the tubes.

3.4 Evaporate off the solvent using the Buchler evaporator at 37°C and 30 atmospheres for 30 minutes.

3.5 Add 200 µL of toluene to each tube; vortex mix, to rinse the tube walls, and then evaporate off the solvent as previously described (3.4).

3.6 Add 200 µL of horse serum reagent to each tube.

3.7 Stopper the tubes and then vortex mix for 20 seconds every minute over a 15 minute period.

3.8 Ultrasonicate the tubes for 40 minutes - 2 hours (this parameter has yet to be optimized); the starting temperature of the bath should be adjusted to 4°C.

3.9 Vortex mix the tubes twice.

3.10 Cover the tubes in aluminum foil and stand overnight at 4°C. This step is optional, but convenient for a lone operator.

3.11 Allow the tubes to equilibrate with room temperature.

3.12 Add 200 µL of antibody diluent to the BLANK tubes.

3.13 Add 200 µL of standardized antiserum (8.16) to each of the remaining tubes, except the TOTAL tubes.

3.14 Re-stopper the tubes and vortex mix.

3.15 Incubate the tubes with shaking at 37°C for 40 minutes.

3.16 Add 7000 cpm of labelled hapten.

3.17 Re-stopper the tubes and vortex mix.

3.18 Incubate the tubes with shaking at 37°C for 60 minutes.

3.19 Transfer the tubes to a 4°C incubator and incubate for 68-72 hours.

4.0 Precipitate the bound radioactivity using the accelerated procedure (9.16).

4.1 Aspirate the supernatant from each tube except the TOTAL tubes.

4.2 Drain the tubes onto absorbent paper for 10 minutes.

4.3 Wipe the inside and lip of each tube using a twirled KIMWIPE^{tm}.

4.4 Re-stopper the tubes and count in the gamma counter using a 10 minute count cycle.

FIGURE 1: Key reagents required for the development of an RIA for the detection of a low molecular weight compound.

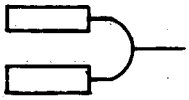
a)  COMPOUND OF INTEREST

b) 

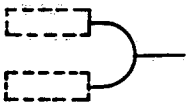
LINKAGE GROUP

c)  = ANTIGEN 'A'

d) ANTISERUM CONTAINS A FAMILY OF ANTIBODIES:

1. TO  Ab_1

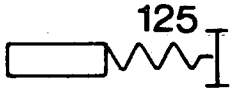
2. TO  Ab_2

3. TO  Ab_3

AND TO VARIOUS SUBCOMPONENTS.

e) REMOVE Ab_2 FROM SERUM BY AFFINITY CHROMATOGRAPHY

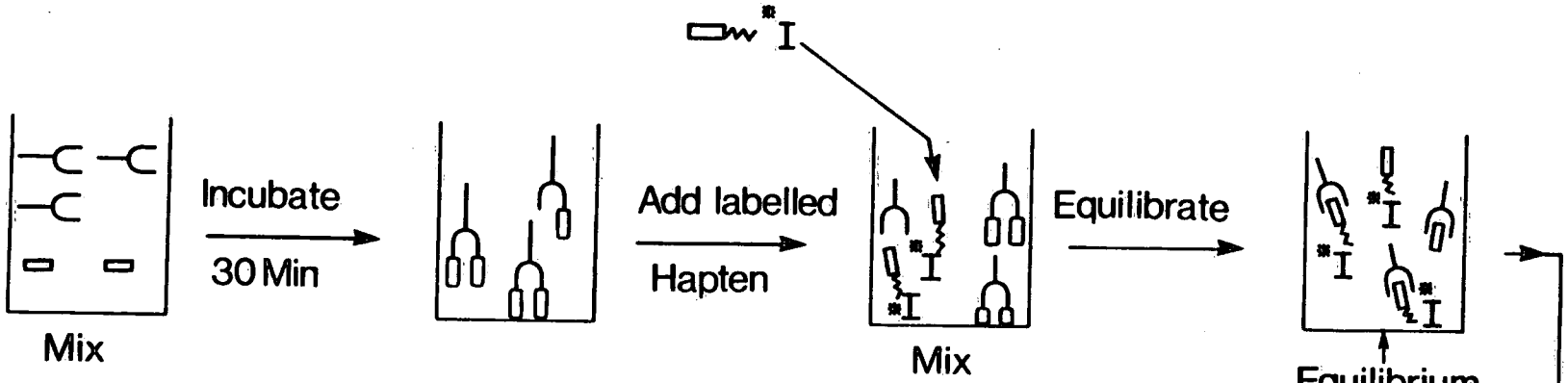
f) LABELLED HAPTEN:



γ EMITTER

$\frac{1}{2}$ LIFE = 2 MONTHS

FIGURE 2: Schematic representation of an RIA for the detection of a low molecular weight compound.



No ▭ will result in 35% binding of ^{125}I