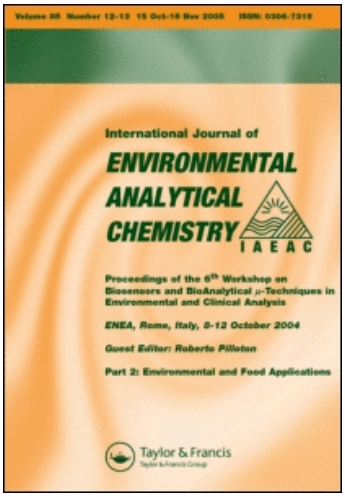

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Radioimmunoassay of PCBs in Milk and Blood

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A radioimmunoassay was developed capable of determining Aroclor 1260 in milk at levels of from 20 to 80 ppb and in blood from 2 to 16 ppb. The values obtained by radioimmunoassay correlate well with those determined by gas–liquid chromatography ($r^2 = 0.96$ for milk and 0.99 for blood) but were lower. Antiserum was produced in rabbits and was specific for 2,2',4,4',5,5'-hexachlorobiphenyl. It cross-reacted with congeners and isomers in Aroclor 1254 and 1260 to the extent that a 20% decrease in binding was observed with 0.1 ng of either mixture. The method requires preliminary cleanup of the extract on alumina and utilizes 25% dimethyl sulfoxide in the assay medium to promote solubilization of the substrates.

KEY WORDS: Radioimmunoassay, PCBs, blood, milk.

INTRODUCTION

Radioimmunoassay (RIA) is a technique which is widely used in clinical chemistry and has been applied to the determination of pesticides[1,2,3,4] and environmental contaminants such as PCBs,[5] chlorinated dibenzodioxins[6] and chlorinated dibenzofurans.[7] Since RIA is often highly selective and offers the possibility of screening large numbers of samples simultaneously it was examined as a method for the routine analysis of milk or blood samples. Luster *et al.*[5] have described an RIA method for PCBs using antisera directed towards 2,3',4,4',5-or 2,2',3,6,6'-pentachlorobiphenyl. While these antisera were relatively specific, they showed little cross-reactivity with Aroclor 1254 resulting in low sensitivity for congeners and isomers of the Aroclor 1254 type. Because 2,2',4,4',5,5'-hexachlorobiphenyl is a major isomer occurring in human tissues,[8,9] we sought an antiserum which would have a high affinity for it as well as similar isomers present in Aroclor 1254 and 1260. To achieve maximum sensitivity, a radioligand of high specific activity and with close structural resemblance to 2,2',4,4',5,5'-hexachlorobiphenyl was synthesized.

MATERIALS AND METHODS

Chemicals. PCB isomers were purchased from RFR Corp., Hope, R.I. Aroclor 1242, 1254 and 1260 were obtained from the Environmental Protection Agency, Triangle Park, N.C. 2,4,5-Trichloroaniline, 1,2-dichlorobenzene, succinic anhydride, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisc. Human serum albumin, 4× crystallized was supplied by ICN Pharmaceuticals Inc., Montreal, Que. and dextran, average M.W. 79,400 by Sigma Chemical Co., St. Louis, Missouri. Charcoal, Darco grade G-60 was from Atlas Chemical Industries Inc., Wilmington, Delaware and gelatin (granular) from Fisher Scientific Co., Fair Lawn, N.J. Freund's Complete Adjuvant was purchased from Difco Laboratories, Detroit, Michigan and sodium iodide-125, carrier free, from Amersham Corp., Oakville, Ontario.

Synthesis: 2,3',4,4',5-Pentachlorobiphenyl. The procedure was as described by Sundström[10] using 2,4,5-trichloroaniline and 1,2-dichlorobenzene as starting materials. The tarry product was purified by chromatography on silicic acid in hexane followed by crystallization from ethanol to give a 13% yield of light pink crystals. Gas–liquid chromatography indicated a purity >99%.

2-Amino-2',4,4',5,5'-pentachlorobiphenyl. Powdered 2,3',4,4',5-pentachlorobiphenyl (3.2 g) was added to a stirred, cold (0°C) 50% solution of conc. HNO_3 in conc. H_2SO_4 (70 ml). The mixture was permitted to come to room temperature slowly warmed to 55°C and maintained at this temperature for 2.5 h, then poured over ice. The solids were filtered, rinsed successively with water and methanol and air-dried. Gas–liquid chromatography of the product indicated 88% nitro derivative and the remainder starting material. The crude product was dissolved in glacial acetic acid (60 ml) at 100°C and reduced by the addition of $SnCl_2 \cdot 2H_2O$ (6.16 g) in conc. HCl (30 ml). The solution was refluxed for 3 h, cooled, poured into ice water, made alkaline with NaOH and extracted with dichloromethane. The extract was washed with 1*N* NaOH, then water, and evaporated to dryness. The resulting oil was transferred to a 3 × 17 cm column of silicic acid in hexane and the column eluted with 20% benzene in hexane (275 ml) which removed pentachlorobiphenyl and nitropentachlorobiphenyl. The aminopentachlorobiphenyl was then eluted with 40% benzene in hexane (400 ml). After evaporation of the solvent, 1.8 g of the pure amine was obtained as an oil.

2-Succinamido-2,4,4',5,5'-pentachlorobiphenyl. Aminopentachlorobiphenyl (456 mg) and succinic anhydride (130 mg) were refluxed for 3 h in toluene (10 ml) containing conc. H_2SO_4 (10 μl). Dichloromethane (25 ml) was added to the cooled reaction solution and the organic phase washed with 1*N* NaOH and water. Acidification of the NaOH wash followed by extraction with dichloromethane yielded a small amount of crystalline material identified as the succinamide by mass spectrometry. Evaporation of the toluene-dichloromethane layer resulted in a solid (375 mg) identified as the succinimide by mass spectrometry. The imide was converted to the amide by refluxing with 0.5*N* NaOH in 50% aqueous methanol for 20 min and the amide recovered, following acidification, by extraction with dichloromethane.

2-[^{125}-Iodo]-*2',4,4',5,5'-pentachlorobiphenyl.* Five microliters of dichloromethane containing 2-amino-2',4,4',5,5'-pentachlorobiphenyl (1.36 μg) were placed in a 6 × 50 mm tube and the solvent removed with a stream of nitrogen. Glacial acetic acid (20 μl) was added and the tube cooled to 0°C. Five microliters of conc. H_2SO_4 containing $NaNO_3$ (0.276 μg) were added and after 10 min at 0°C, $Na^{125}I$ (10 mCi, carrier free) added in 20 μl of water. The tube was brought to room temperature, then placed in an 80°C block for 15 min. The contents of the tube were transferred to a 15 ml centrifuge tube using a Pasteur pipette and 2 ml of hexane, and the hexane phase shaken with 10% $K_2S_2O_5$ (10 ml) and 1*N* NaOH (0.5 ml). The hexane layer was removed and added to a 2 g column of 2% deactivated Florisil in hexane. The column was eluted with hexane (12 ml) and the eluate diluted to 25 ml with hexane. Gas–liquid chromatography indicated an 80% conversion to the iodo derivative, the remainder consisting of 15% pentachlorobiphenyl and 5% unknown. The specific activity of the radioligand was determined to be 2073 mCi/μmole. For the assay, an aliquot of the hexane solution was taken to dryness and redissolved in an appropriate volume of dimethyl sulfoxide.

Immunogen. The immunogen consisted of succinamido pentachlorobiphenyl coupled to human serum albumin. Human serum albumin (70 mg) was dissolved in water (2 ml) and an aqueous solution of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (57 mg; 1.0 ml) added. A solution of succinamido pentachlorobiphenyl (43.9 mg) in dilute NaOH, pH 11–12 (5 ml) was added immediately and the pH adjusted to 7.0 with 1*N* HCl. The mixture was stirred overnight at room temperature, then dialyzed at 4°C against several changes of 0.01 M Na_2CO_3 followed by distilled water and lyophilized. It was estimated by UV spectrophotometry that 1 molecule of albumin contained

26 mole of hapten. For injection into rabbits, the immunogen (3.5 mg) was dissolved in 0.14 N NaCl (1.1 ml) and mixed with Freund's Complete Adjuvant (2.4 ml) to give an emulsion containing 1 mg/ml of immunogen.

Instruments. Gas–liquid chromatography (GLC) was performed on a Hewlett Packard 5700A fitted with a ^{63}Ni electron capture detector and 6′ × 4 mm i.d. glass column packed with 3% SE-30 on 100–120 mesh Chromosorb W (AW). The column was operated at 225°C, injection port at 250°C and detector at 300°C. The carrier gas was argon:methane (95:5) at a flow rate of 30 ml/min. Samples were quantitated by comparison of the heights of peaks 7, 8, 10, 11 and 13 of Aroclor 1260, according to the numbering system of Reynolds.[11]

Mass spectrometry was carried out on a Varian MAT 311A using 70 eV electron impact ionization.

Iodide-125 was counted on a Beckman Gamma 8000 scintillation counter using 1 min counts at an efficiency of 77%.

Buffers. Phosphate buffered saline (PBS) was prepared by dissolving 20 mmole NaH_2PO_4, 140 mmole NaCl and 3 mmole NaN_3 in distilled water and adjusting the pH to 7.15 with NaOH before dilution to 1 l. Antiserum diluent consisted of 0.1% gelatin in PBS. Charcoal suspension contained 1 mg/ml charcoal and 0.1 mg/ml dextran in 25% dimethyl sulfoxide in antiserum diluent.

Antisera. New Zealand rabbits were injected subcutaneously at multiple sites with 0.5 ml each of an emulsion of immunogen (1 mg/ml) in Freund's Complete Adjuvant. Booster injections were given at monthly intervals. Blood was collected from the marginal ear vein 1 week after the booster injections and serum prepared. Useful titers were obtained 3 months after the initial injection. Antiserum was stored frozen (−20°C) in 100 μl aliquots.

Analysis

Sample extraction. The extraction procedure was similar to that of Van Renterghem and Devlaminck.[12] Human or cows' milk (1.0 g) was placed in a 15 ml centrifuge tube and extracted by shaking for 1 min with 14 ml of acetone:hexane (2:1). Milk solids were sedimented by centrifugation and the liquid decanted into a 60 ml separatory funnel. Water (5 ml) was added and the phases mixed by gentle shaking. The hexane layer was recovered and evaporated to dryness with a stream of nitrogen in a 15 ml conical tube.

Whole blood (0.5 g) was weighed into a 15 ml centrifuge tube and extracted by shaking with 10 ml of acetone:hexane (2:1). Solids were removed by centrifugation and the liquid decanted into another 15 ml centrifuge tube where it was partitioned by gentle shaking with water (5 ml). The phases were separated by centrifugation and the hexane layer removed and evaporated in a 15 ml conical tube with nitrogen.

Cleanup. Residue from milk or blood extracts was taken up in hexane (1.0 ml) and applied to a 2.0 g column of neutral alumina (activity I) prepared in hexane in a 8 mm i.d. column (Kontes, size 22). The tube was rinsed with a further 0.5 ml of hexane and elution commenced with freshly prepared 2% dichloromethane in hexane. The first 10 ml of eluate were collected and triplicate aliquots (0.10 ml for milk; 1.0 ml for blood) taken to dryness in 12 × 75 mm tubes for radioimmunoassay. A 5.0 ml aliquot was taken to dryness for gas–liquid chromatographic determination of PCBs.

Radioimmunoassay. Standards consisting of 0, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 ng of Aroclor 1260/50 μl dimethyl sulfoxide were placed in triplicate 12 × 75 mm tubes. Fifty microliters of dimethyl sulfoxide were added to each sample tube and 50 μl of radioligand containing approximately 40,000 cpm of ^{125}iodopentachlorobiphenyl added to both sample and standard tubes. The samples were placed in a Bransonic 220 ultrasonic cleaner for 15 min to dissolve the residue. Diluent (100 μl) was added to all tubes followed by a 1:3000 dilution of antiserum (200 μl). After mixing on a vortex mixer, the tubes were incubated at 4°C overnight. Cold (4°C) charcoal suspension (1.0 ml) was added and the tubes shaken on a reciprocal shaker for 5 min. The charcoal was sedimented by centrifugation at 1200 × g for 10 min and the supernatant decanted into 12 × 75 ml polystyrene tubes for counting.

Nonspecific binding was determined by substituting diluent for diluted antiserum. Serum from non-immunized rabbits bound the radioligand an amount equal to that of the diluent when diluted 1:3000. A standard curve of cpm bound ligand was plotted against the ng of Aroclor 1260 added and the samples quantitated by comparison of the cpm obtained with those of the standard curve.

RESULTS AND DISCUSSION

It has been noted by Luster *et al.*[5] that a major difficulty in developing an RIA for chlorinated hydrocarbons is the solubilization of these compounds in a manner compatible with binding to the antibody. These

authors employed nonionic detergents such as Cutscum for solubilization in a double antibody technique. In the course of method development, we observed that dimethyl sulfoxide was an effective solvent for PCBs and was superior to 1% Cutscum in permitting a higher degree of binding of ligand to antibody.

Charcoal was used to separate bound and unbound ligand because of its simplicity and speed relative to other methods. In the absence of dimethyl sulfoxide, a charcoal suspension of 8 mg/ml was required to produce nonspecific binding of 5–7%. The incorporation of 25% dimethyl sulfoxide in the suspension permitted the charcoal concentration to be lowered to 1 mg/ml while maintaining the same level of nonspecific binding, but resulted in a 10-fold increase in the sensitivity of the assay.

From the standard curve shown in Figure 1, it is evident that the RIA is easily capable of detecting 0.1 ng of Aroclor 1260. With gelatin as a diluent, nonspecific binding was approximately 5–7% of the added counts, while the use of albumin or γ-globulin resulted in nonspecific binding several times higher and an accompanying loss of sensitivity of the assay.

The assay is most specific for the 2,2′,4,4′5,5′ isomer as shown by the data in Figure 2. As observed by Luster *et al.*,[5] the nitrogen atom of the amino group of the hapten acts as a chlorine atom for the purpose of antibody binding. The addition of an *ortho* chlorine, as in the 2,2′4,4′5,5′,6 congener decreases binding slightly whereas deletion of it as in the 2,4,4′5,5′ congener causes a 10-fold reduction in binding. Deletion of both *ortho* chlorines from the most active structure gives the toxic[13] 3,3′4,4′ congener which is bound feebly. The addition of *meta* chlorines to give the 2,2′,3,3′,4,4′,5,5′ congener decreases the binding significantly. The absence of chlorine atoms in one ring, even if the other contains the preferred 2,4,5 configuration, causes a dramatic reduction in binding. This effect has also been noted with the activity of PCBs as microsomal enzyme inducers.[14]

Aroclor 1260 and 1254 produce similar standard curves, the latter being bound 10% less than 1260. However, with Aroclor 1242, 1.3 ng are required to produce a 20% decrease in binding of radioligand. Thus, the assay will measure either Aroclor 1260 or 1254 in a sample, but is sensitive to less than 10% of the Aroclor 1242, if present. Biphenyl, hexachlorobenzene, p,p′-DDE or heptachlor did not affect the assay at levels up to 1 μg.

The recovery of Aroclor 1260 from fortified human or cows' milk is given in Table I. The values obtained by the RIA method correlate well ($r^2 = 0.96$) with those determined by GLC, although the average percentage recovery by RIA was 74% compared to 99% by GLC. The mean interassay CV was 13% for analyses performed on 5 different days.

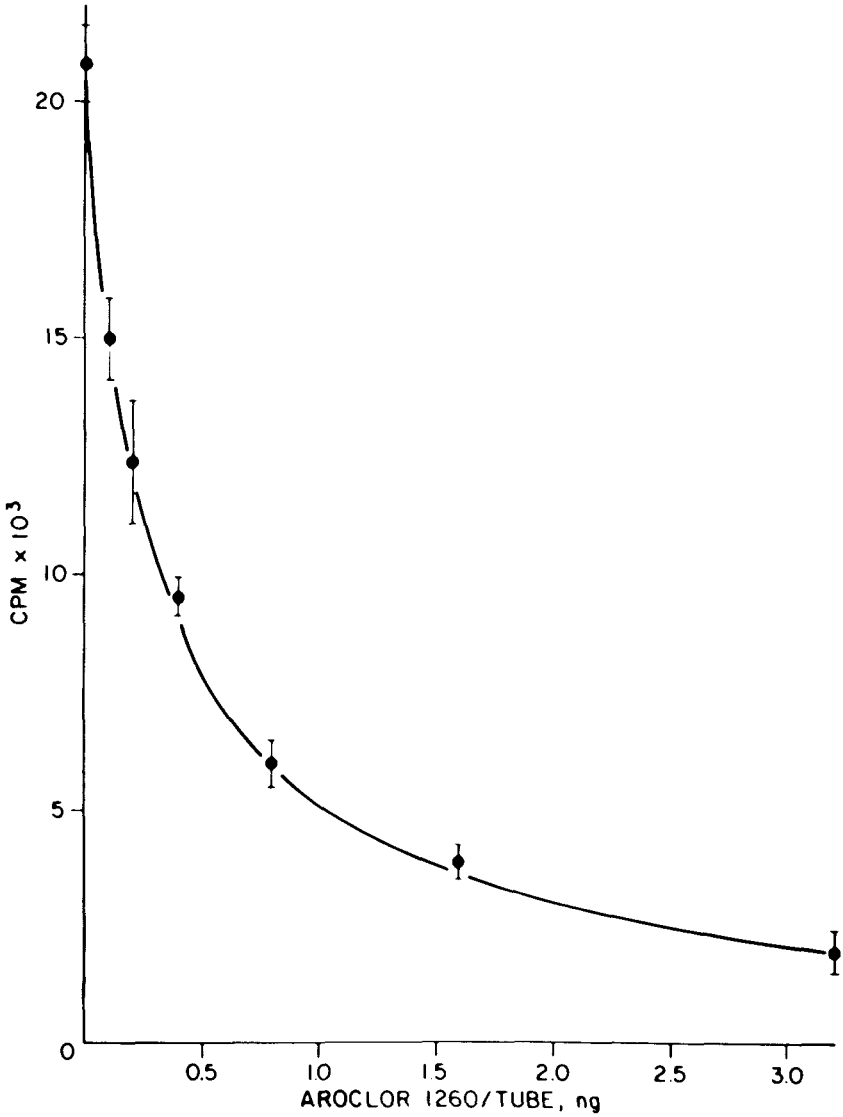

FIGURE 1 Standard curve for the radioimmunoassay of Aroclor 1260. Cpm are counts of radioligand bound, corrected for nonspecific binding. Standard deviation is indicated by the vertical lines.

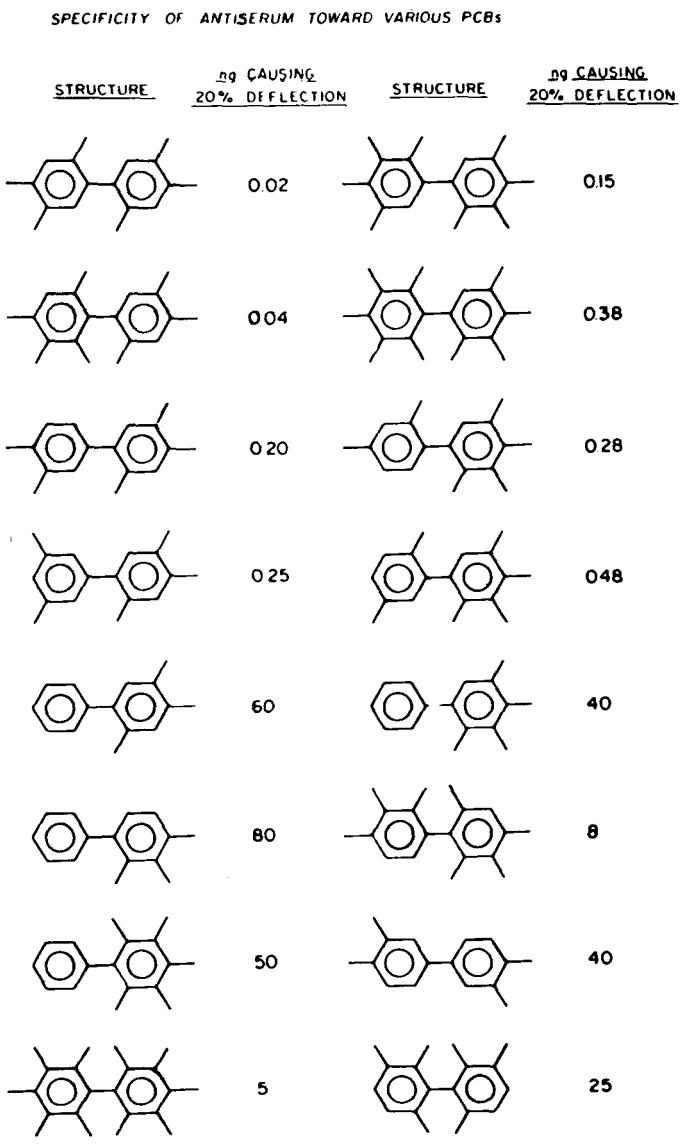

FIGURE 2 Structures of various PCBs and their relative binding by antiserum directed toward 2-acetamido-2,4,4′,5,5′-pentachlorobiphenyl. Relative binding is measured by the amount of PCB required to displace 20% of the radioligand bound in the absence of inhibitor.

TABLE I

Recoveries[a] of Aroclor 1260 from human and cows' milk

Aroclor 1260 added, ppb	Aroclor 1260 found, ppb			
	Human milk		Cows' milk	
	RIA	GLC	RIA	GLC
0	16	16	3	0
20	29	35	15	21
40	47	55	36	40
80	71	95	74	76

[a]Results are the average of determination on 2 samples.

Data from human blood fortified with various levels of Aroclor 1260 are given in Table II. As with milk, the values obtained by GLC are higher than those by RIA, averaging 96% compared to 79% by RIA. Good correlation between the two methods was evident ($r^2 = 0.99$). A mean interassay CV of 12% was calculated for analyses carried out on 4 different occasions.

TABLE II

Recoveries[a] of Aroclor 1260 from human blood

Aroclor 1260 added, ppb	Aroclor 1260 found, ppb	
	RIA	GLC
0	2.7	1.9
4	5.3	5.8
8	9.1	9.4
16	17.4	17.4

[a]Results are the average of determinations on 2 samples.

RIA is a rapid method of screening multiple samples for PCB content. It requires cleanup as extensive as that for GLC analysis to remove traces of lipid which, if present, produce false negative values. After cleanup, samples can be processed at a rate of approximately 75/day/technician. Sufficient material remains after RIA to determine congener and isomer content on those samples designated as positive.

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