Development of a Microcolumn Radioimmunoassay for Screening of Polychlorinated Biphenyls in Milk and in Animal Fats

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6-[^{125}I]Iodo-3,3′,4,4′-tetrachlorobiphenyl and *anti*-3,3′,4,4′-tetrachlorobiphenyl-6-azo–serum were used for the development of a Spheron microcolumn radioimmunoassay (RIA) which permits a screening detection of PCBs in cleaned up extracts from fat materials. The assay was negligibly sensitive to chlorinated pesticides and further aromatic and cyclic hydrocarbons. The developed method detected in the test tube 0.1 ng of Delor 104, 105, or 106 (the corresponding formulations are Aroclor 1248, 1254, or 1260) and exhibited considerably lower sensitivity to Delor 103 (Aroclor 1242). The values obtained by means of RIA were comparable with those determined by GC in the detection range 0.088–5.58 mg of Delor 106 kg^{-1} of fat. Intra-assay variation coefficients in samples containing 0.5, 1.25, and 2.8 mg of Delor 106 kg^{-1} of fat were 21.2 (n = 40), 9.0 (n = 17), and 11.9% (n = 23), respectively; interassay coefficients for samples with concentrations of 0.5 and 2.8 mg of Delor 106 kg^{-1} of fat were 23.9 (n = 37) and 24.6% (n = 30), respectively. A specific application of the method for monitoring a contaminated cow herd is presented.

From different overviews it is evident that PCBs are the most ubiquitous industrial pollutants in the global ecosystem, representing potential health risks (Safe, 1984, 1989; McFarland and Clarke, 1989; Silberhorn et al., 1990). PCB production in the world peaked in 1970 and gradually decreased in the 1970s, when a number of countries either prohibited their production or limited their use (Silberhorn et al., 1990). In Czechoslovakia the production of PCBs was prohibited only in 1984. Two years later the manufacture of condensers with PCB filling was terminated as was the manufacture of paints with PCB content. It is known that PCBs are bioaccumulated in the food chain and their residues are found predominantly in adipose tissues, milk, and blood. Because of the harmful biological effects of PCBs on human and animal organisms, there exists a need for routine screening, particularly for the presence of residues in the environment and in food samples. The usual practice in regulatory evaluation of PCBs in environmental samples is to quantify either the PCB total or the total related to Aroclor equivalents (FDA, 1979). Recently, efforts have been made to introduce congener-specific analysis (Safe et al., 1985; McFarland and Clarke, 1989; Mes et al., 1990). Analytical techniques are based predominantly on GC and/or HPLC with different systems of detection (McFarland and Clarke, 1989; Lindsey, 1989) and less frequently on a combined GC/MS method (Alford-Stevens et al., 1986).

In the past decade, immunochemical methods have been used for the detection of different trace chemical contaminants in the environment as a relatively cheaper alternative to conventional chemical methods (Vanderlaan et al., 1988). Unlike many other biologically important compounds, the quantitation of PCBs is substantially more difficult due to (1) a high molecular heterogeneity of the polychlorinated analyte, (2) the heterogeneity of the toxicity of individual congeners, (3) the low solubility of the analyte in water, (4) the need to clean up the extracted samples before the analysis, and (5) the adsorption affinity of PCBs on the walls of the working materials.

The radioimmunoassay (RIA) presented in this paper was able to detect a broad spectrum of polychlorinated biphenyls that are regularly deposited in the adipose tissues of animals and humans (Mes and Lau, 1983). The method was applied to extracts of milk and adipose tissues cleaned up on Florisil or by acid hydrolysis.

MATERIALS AND METHODS

Chemicals and solvents, if not stated otherwise, were purchased from Lachema (Brno, Czechoslovakia). The serum bovine albumin and Al-Span-Oil adjuvants were obtained from Sevac (Prague, Czechoslovakia) and pig thyroglobulin (TG) and Norit A from Serva (Heildelberg, Germany). Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem-Behring Co. Dextran T 70 and Sephadex G-25 were from Pharmacia Fine Chemicals (Sweden), and Florisil, particle size 60–100 mesh, was from Merck (Darmstadt, Germany). Spheron 40 was a product of Lachema. Standards Delor 106, 105, 104, and 103 (content of chlorine being 60, 54, 48, and 42%, respectively) were products of Chemko (Strážské, Czechoslovakia) and obtained as a present from Dr. Dubský from the Institute of Forensic Medicine (Brno, Czechoslovakia). These formulations are analogs of Aroclor 1260, 1254, 1248, and 1242, respectively. 2-, 4,4′-, 2,4,5-, and 3,3′,4,4′-chlorinated biphenyls with minimum 99% Pestanal were purchased from Riedel-de Haen (Hannover, Germany). Standards of 3,3′,4,4′-, 3,3′,4,4′,5-, and 3,3′,4,4′,5,5′-chlorinated congeners were obtained as a present from Dr. S. Tanabe, Ehime University (Japan), the same as 2,2′,4,5,5′-, 2,2′,4,4′,5,5′-, and 2,2′,3,3′,4,4′,5,5′-chlorinated congeners were obtained from Dr. S. Safe, University of Texas. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was purchased from Supelco. Na^{125}I, specific radioactivity 4500 MBq/mL, was purchased from the Institute of Isotopes of the Hungarian Academy of Sciences (Budapest, Hungary). Phosphate buffer, pH 7.0 (assay buffer), contained 1.56 g of $NaH_2PO_4 \cdot 2H_2O$, 8.16 g of NaCl, 0.2 g of NaN_3, and 1 g of gelatin (Oxoid) in 1 L of deionized water; the resulting pH was adjusted by means of NaOH (4 mol/L). Borate–carbonate buffer, pH 9.4, contained 1.892 g of Na_2CO_3 and 3.401 g of $Na_2B_4O_7 \cdot 10H_2O$ in 1 L of deionized water. Glass tubes (Sklovina, Unihost) were obtained from glassworks Kavalier (Czechoslovakia). Plastic (polypropylene) tubes were purchased from Chirana (Czechoslovakia).

Instrumentation. The determination of melting points was made on a melting point apparatus obtained from Franz Küstner Nacht. Wissenschaftliche Apparate (Dresden, Germany) without correction of values. NMR spectra were obtained with a WP 80 SY Bruker spectrometer (Germany). Absorption spectra were determined on an automatic registration Varian spectrophotometer, type Superscan 3. Radioactivity was measured on a NE-1600 Nuclear Enterprises Ltd. (Great Britain) computer. Gas chromatography was made on a Varian 2700 chromatograph with electron capture detector and a recorder. A glass column

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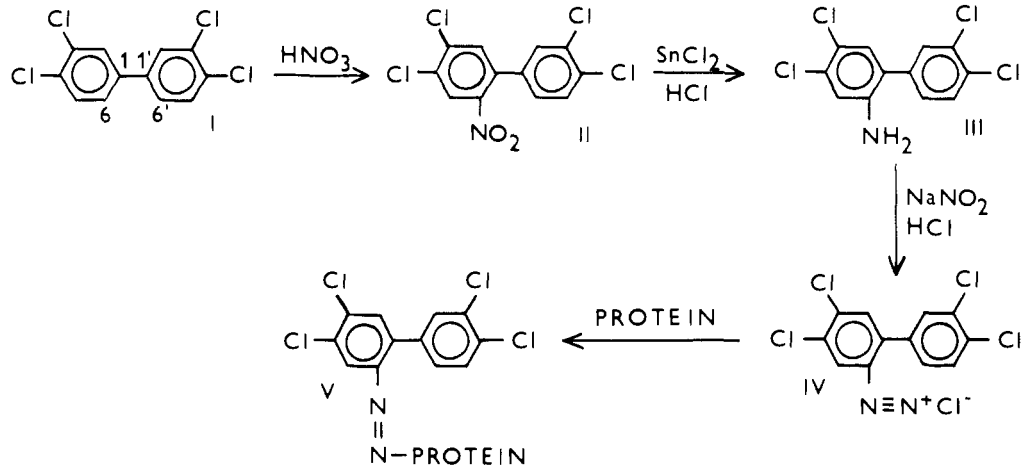

Figure 1. Synthesis of 3,3′,4,4′-tetrachlorobiphenyl-6-azo–protein immunogens.

was used, 2-m length, with 5% DC 200 packing on Varaport 30. Conditions were as follows: detector temperature, 300 °C; columns, 220 °C; injector, 255 °C; N_2 flow rate, 40 mL/min. For chromatogram evaluation peaks of Delor 106 were used with the following retention times (DDE = 100): RRT_{DDE} 1/146, 2/174, 3/203, 4/280, 5/331.

Synthesis of Immunogens. As a starting substance for the synthesis 3,3′,4,4′-tetrachlorobiphenyl (I) was used, which in the first stage was converted to a nitro derivative (II) (Figure 1). By reduction of the nitro group the amino derivative (III) was obtained, which was diazoted in an acid environment, forming 3,3′,4,4′-tetrachlorobiphenyldiazonium chloride (IV). The resulting azoproduct (V) was obtained by an azocoupling reaction of this diazonium salt with proteins.

6-Nitro-3,3′,4,4′-tetrachlorobiphenyl (II). The nitration of tetrachlorobiphenyl (I) was carried out by a modified method according to Chaudhary and Albro (1978). Acetanhydride (1.1 mL, 0.01 mol) cooled to 0 °C was mixed with fuming HNO_3 (0.8 mL, 0.02 mol) cooled to the same temperature, and to this mixture was slowly added tetrachlorobiphenyl (2.5 g, 0.086 mol). The temperature of the reaction mixture increased to 50 °C after 2 h of stirring at room temperature, and the mixture was left at that temperature for 30 min in a water bath. Then the mixture was poured into water with ice (about 20 g/50 mL), and the solid fraction was filtered off on a fritted glass, washed with about 50 mL of cold water, and dried under an infrared lamp. Yellow powder was obtained, which was recrystallized twice from hot ethanol, $C_{12}H_5Cl_4NO_2$: N, 3.9%. Found: N, 4.15%. The ^1H NMR spectrum of the product dissolved in $CDCl_3$ with tetramethylsilane as internal standard, measured at 80.13 MHz, gave the following signals: H(3) s, 8.08 ppm; H(5′) d, 7.51 ppm; H(6) s, 7.50 ppm; H(2′) d, 7.39 ppm; and H(6′) q, 7.09 ppm.

6-Amino-3,3′,4,4′-tetrachlorobiphenyl (III). Reduction of 6-nitro-3,3′,4,4′-tetrachlorobiphenyl was carried out according to the method of Chaudhary and Albro (1978). To the 6-nitro derivative (1 g, 0.003 mol) was added $SnCl_2$ (1.7 g) in concentrated HCl (7 mL). The mixture was boiled for 3 h under reflux and then left standing overnight at room temperature. Alkalization of the yellow solution with NaOH (17 g NaOH/100 mL) led to the formation of a milky white solution, which was extracted three times with 20 mL of diethyl ether. Ether extracts were poured out, shaken twice into 20 mL of distilled water, and dried over anhydrous Na_2SO_4. After vacuum evaporation of ether, brown powder was obtained, which was recrystallized from hot ethanol. Product (0.8 g) was obtained with 87% yield, mp 81 °C. Elementary anal. calcd for $C_{12}H_7Cl_4N$: N, 4.50%. Found: N, 4.35%. The ^1H NMR spectrum of the product dissolved in $CDCl_3$ with tetramethylsilane as internal standard, measured at 80.13 MHz, yielded the following signals: H(3) s, 6.85 ppm; H(6) s, 7.13 ppm; H(2′) d, 7.50 ppm; H(5′) d, 7.53 ppm; and H(6′) q, 7.23 ppm.

3,3′,4,4′-Tetrachlorobiphenyldiazonium Chloride (IV). Amino derivative (79.5 mg, 0.26 mmol) was added to water (4.5 mL) and concentrated HCl (0.34 mL, 3.96 mmol), and the suspension formed was intensively stirred. $NaNO_2$ (1 M; 1 mL) was added dropwise under stirring in an ice bath over the course of about 10 min. Another addition of concentrated HCl (0.5 mL) into the diazotation mixture resulted in the formation of white opalescence with a lower fraction of solid state. The diazonium salt (IV) formed was cooled in an ice bath until use.

Immunogens (V). The reaction of diazonium salt with proteins was performed in borate–carbonate buffer, pH 9.4, as follows: (a) To the solution of albumin (172.5 mg/12.5 mL of buffer, 2.5×10^{-3} mmol) was added diazoted mixture (3 mL) dropwise under stirring over the course of about 30 min. The mixture was maintained within pH 8–10 by adding 1 M NaOH during the addition of the acid solution of diazonium salt. Then the reaction mixture was adapted to pH 9.5. Autocoupling products were removed by dialysis against distilled water with subsequent gel filtration on Sephadex G-25 (Fránek and Hruška, 1983). The product (immunogen) was lyophilized and kept at −20 °C. (b) To the solution of thyroglobulin (206.25 mg/11.25 mL of buffer, 3.1×10^{-4} mmol) was added diazoted mixture (1.5 mL) under conditions as in (a). The resulting solution of azothyroglobulin was stained red. (c) To the solution of hemocyanin (187.0 mg/11.25 mL of buffer, 6.2×10^{-5} mmol) was added 1.5 mL of diazoted mixture under conditions as in (a). In the course of the reaction the solid fraction precipitated and was removed by centrifugation.

Antisera. Immunization of 12 Californian white rabbits and 3 goats was carried out by intradermal application of immunogens after emulgation in Al-Span-Oil adjuvants. One immunization dose contained in rabbits about 80 μg of immunogen applied in doses of 100 μL to 10 sites of the back region and 5 sites of the lumbar region. In goats the immunogens were applied to 50 sites of the back region with an immunization dose of 250 μg. Immunizations were repeated on the 14th day and then after every 30 following days over the course of at least 4 months. The plasmas were obtained about the 10th day following the last immunization.

6-[^{125}I]Iodo-3,3′,4,4′-tetrachlorobiphenyl. Radioiodine was incorporated into the biphenyl skeleton after the diazonium cation was split off according to a slightly modified method of Newsome and Shields (1981). Twenty microliters of 6-amino-3,3′,4,4′-tetrachlorobiphenyl in hexane (0.7 mg/5 mL) was added into a glass tube with ground stopper. After evaporation of the solvent using a stream of nitrogen, glacial acetic acid (20 μL) was added into the tube; 10 μL of concentrated H_2SO_4 containing $NaNO_2$ (1 mg/20 mL) was added to the reaction mixture under cooling in an ice bath, and after 10 min with occasional stirring, 30 μL of Na^{125}I (about 100 MBq) was added. The mixture was taken away from the ice bath and put for 20 min in a water bath of 80 °C with occasional stirring. At room temperature hexane (2 mL) was added to the iodination mixture, and the mixture was shaken for 2 min with 10% $Na_2S_2O_5$ (10 mL) and 1 M NaOH (0.5 mL). By means of a Pasteur pipet the hexane phase was applied to the column (17 × 1 cm) filled with deactivated Florisil (about 2 g). The column was eluted with hexane (12 mL), and the radioactive effluent was evaporated under a stream of nitrogen. The evaporate was dissolved in redistilled DMSO and used for radioimmunoassay.

Spheron Microcolumn Radioimmunoassay. The separation of free and bound radioactivities in the assay mixture was performed on microcolumns according to the modified method described in our earlier paper (Fránek et al., 1988). Microcolumns were prepared in the following way: Spheron 40, particle size 0.025–0.040 mm, was left overnight to swell in assay buffer with occasional stirring. The amount of buffer was adapted in such a way as to make the ratio of the sediment and the liquid phases be 1:1 (v/v); 0.5 mL of this suspension was taken under stirring and pipetted into the 1-mL plastic tip of a micropipet (Chirana, Czechoslovakia) which formed the jacket of the microcolumn with inserted silica wadding in the lower part. The microcolumns located in the eluate tubes in a rack were inserted at least 1 h before use into a refrigerator and left to drip. Reaction mixtures were prepared in glass tubes as follows: 50 μL of standard Delor 106 in hexane of concentrations 0.125, 0.25, 0.5, 1.0, 2.0, and 5.0 ng/50 μL or the sample in hexane was pipetted into tubes and was left to evaporate at room temperature. Then 100 μL of the tracer in DMSO (about 30 000 cpm) and 300 μL of the antibody diluted in the assay buffer were added. The mixtures were shaken in a vortex and incubated in a refrigerator overnight. After incubation, the mixtures were transferred by means of a pipet with a 1-mL plastic tip into precooled microcolumns in 300-μL aliquots at room temperature, and the microcolumns were again inserted into a refrigerator. After the reaction mixture was soaked into Spheron, the microcolumns were eluted with 0.7 mL of assay buffer and returned to the refrigerator. Eluted radioactivity was measured directly in the tubes. For routine screening, animal fat with a defined content of Delor 106 (5.58 mg kg^{-1} fat) was used as calibration standard. The fat standard was weighed before each analysis and worked up by the method of acid hydrolysis, and the solution thus obtained was used for the calibration by gradual dilution with hexane. Fifty microliters of the calibration standard was pipetted into glass tubes with subsequent evaporation of the solvent at room temperature.

Measurement of the Adsorption of PCB on Assay Materials. As the radioindicator for adsorption experiments, 6-[^{125}I]-iodo-3,3′,4,4′-tetrachlorobiphenyl in hexane was used in the concentration corresponding to about 50 000 cpm/tube. In model experiments losses of radioactivity were measured after the interaction of the liquid phase with the tube. A radioindicator in hexane and Delor 106 in hexane were added into the test tubes in the amounts of 0.25 and 10 ng. After evaporation of the solution under a gentle stream of nitrogen, 1 mL of hexane or DMSO was added to the tubes. The tubes were vigorously shaken on a vortex and left to stand. After removal of the solvent, the adsorbed activity was measured directly in the tubes. PCB adsorption under assay conditions was determined as follows: The radioindicator and Delor 106 in hexane were evaporated in the tubes, and DMSO (100 μL), buffer, or diluted antiserum (300 μL) was added. The mixtures were vortexed and incubated in a water bath at different temperatures and for different times. Adsorbed radioactivity was measured after the removal of the liquid phase directly in the tubes. The testing of PCB adsorption after solubilization of the evaporates and subsequent ultrasound treatment was carried out using an ultrasonic cleaner UG/320 TA (Tesla).

Sample Preparation. *Extraction of Milk Fat.* Milk (40 mL) was centrifuged, and milk fat was spread with anhydrous Na_2SO_4, dissolved in petroleum ether, and filtered through the same anhydrous Na_2SO_4. Petroleum ether was evaporated, and the fat obtained was purified according to methods described below.

Extraction of Animal Fats. Suet or fat was cut into small pieces, spread with anhydrous Na_2SO_4, and poured over with petroleum ether. After occasional stirring over the course of at least 4 h, the organic phase was filtered through anhydrous Na_2SO_4 into a flash and evaporated.

Cleanup on Florisil Column. The column (2 × 30 cm) filled with about 10 g of activated Florisil was washed with petroleum ether (50 mL) and dichloromethane (50 mL). About 0.2 mg of extracted fat in petroleum ether was applied to the column, and after soaking into the column, it was eluted with petroleum ether (250 mL) with the addition of 6% diethyl ether or dichloromethane. The eluate was evaporated and the evaporate dissolved in hexane prior to analysis.

Cleanup by Acid Hydrolysis. Extracted fat (about 0.2 g) was exactly weighed into a 20-mL vessel and dissolved in petroleum ether (10 mL). Concentrated H_2SO_4 (1 mL) was added to the flask and the mixture stirred with a glass rod; 5 mL of the upper organic layer was taken into a tube with ground stopper, and petroleum ether (5 mL) and concentrated H_2SO_4 (1 mL) were added. The tube was closed with a stopper, shaken vigorously several times, and left to stand for at least 15 min. In the lower part of the tube there was a layer of acid with degraded fat. The organic phase (5 mL) was taken into a flask and evaporated. The dry evaporate was dissolved in hexane and used for analysis. In this way one-fourth of the original weight of fat was used for the analysis.

RESULTS

Analyte and Immunogens. As the starting hapten for the conjugation we used the 3,3′,4,4′ congener (Figure 1), although our intention was not the detection of its supertrace presence in technical PCB formulations or in the environmental samples. The practical importance of this immunogenic conjugate is its ability to evoke antibody formation with broad cross-reactivity among tetra–heptachlorinated biphenyls. The immunochemical aspects of this phenomenon are expanded upon under Discussion. The obtained hapten–azo–protein conjugates were characterized by the absorption of light in the visible region of the spectrum. 3,3′,4,4′-Tetrachlorobiphenyl-6-azo–TG exhibited a symmetric flat peak λ_{max} = 500 nm, ϵ = 22.0 × 10^4 L mol^{-1} cm^{-1} in 0.1 M NaOH, the disappearance of absorption taking place in this region in phosphate buffer at pH 7.0. Similar spectral characteristics were obtained with the albumin and the hemocyanin immunogens.

Antisera. The immune response to 3,3′,4,4′-tetrachlorobiphenyl-6-azo–protein immunogens was monitored in the blood of immunized animals over the course of immunization by measuring the antiserum titer, i.e., the dilution at which 50% of the radioactivity of the iodine tracer is bound to this antiserum. Albumin and thyroglobulin immunogens were applied to groups of five rabbits; hemocyanin immunogen was injected into three rabbits. In all rabbits albumin immunogens produced antisera with titers of about 1:100, which were not tested further. Immunization with thyroglobulin and hemocyanin immunogens resulted in titers higher than 1:400. In goats antisera with a titer of 1:600 were obtained by the albumin immunogen and with a titer of 1:1000 by both the thyroglobulin and the hemocyanin immunogens. A more detailed characterization was carried out on the rabbit antiserum raised by the thyroglobulin immunogen (RTG-3), the goat antiserum against the thyroglobulin immunogen (GTG-1), and the goat antiserum against the hemocyanin immunogen (GHC-1).

Optimization of the Assay Conditions: PCB Adsorption on Glass and Polypropylene. The studies included the determination of PCB losses after contact with the walls of materials in which the evaporation of solvents is performed, as well as the solubilization of evaporates and their own assessment (see Measurement of the Adsorption of PCB on Assay Materials). In the first model experiment performed in glass tubes the decrease in radioactivity of the radioindicator in the solvent was found after solubilization of Delor 106 and subsequent standing of 10 min. The recovery of radioactivity after solubilization in DMSO was 70.5 ± 6.5% and was 99.5 ± 0.3% after solubilization in hexane. On the other hand, in Delor 106 evaporated from hexane in polypropylene tubes virtually all radioactivity was adsorbed on the walls of these tubes, if the solubilization was performed in DMSO at different times of standing at room temperature. In further experiments we followed the adsorption of radioactivity on the walls of tubes under assay conditions. In 25% DMSO in assay buffer without the presence of the

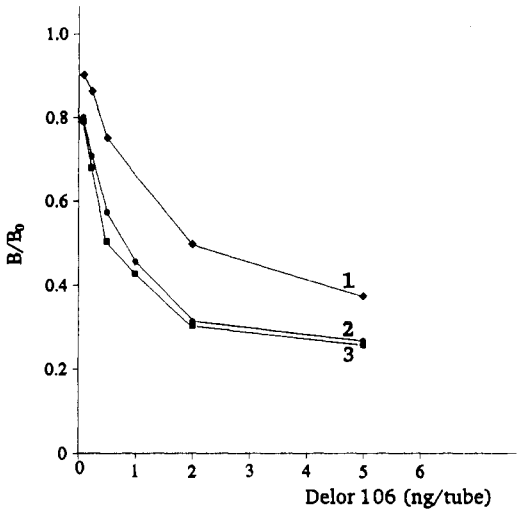

Figure 2. Displacement curves for antisera GTG-1 (1), GHC-1 (2), RTG-3 (3), and Delor 106 as a standard. Antisera bound about 40% iodinated tracer (total activity in tube 20 000 dpm) at final dilutions of 1:1000 (1), 1:1000 (2), and 1:600 (3).

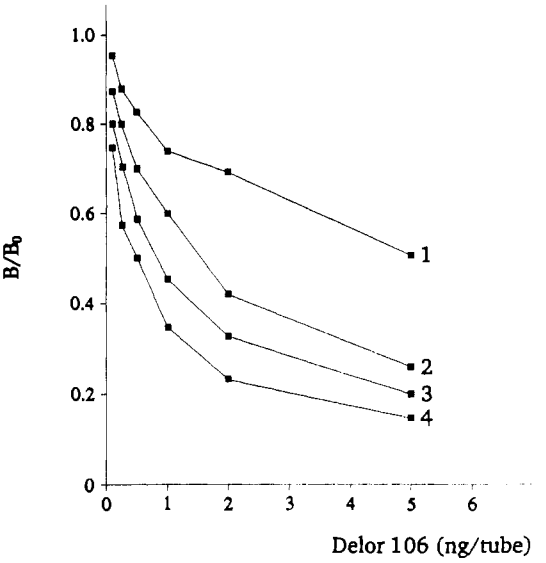

Figure 3. Displacement curves for antiserum GHC-1 and Delor 103 (1), Delor 104 (2), Delor 105 (4), and Delor 106 (3). The antiserum bound about 40% of iodinated tracer (total activity in tube 20 000 dpm) at a final dilution of 1:1000.

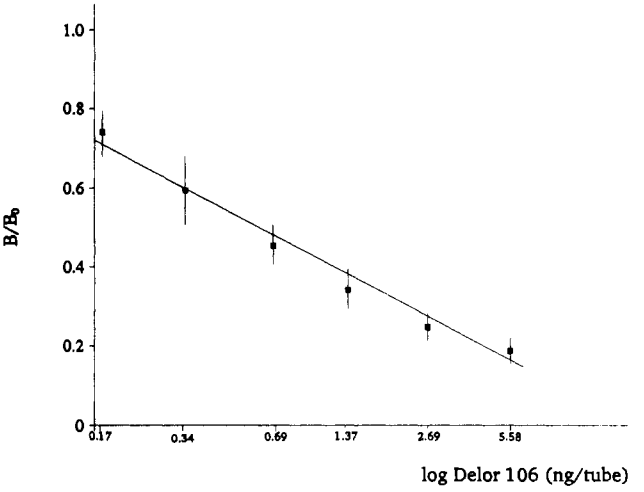

Figure 4. Calibration curves for antiserum GHC-1 and fat standard with defined Delor 106 content. The antiserum bound about 40% of iodinated tracer (total activity in tube 20 000 dpm) at a final dilution of 1:1000. This curve was obtained from 18 curves in the course of 8 weeks. The fat material was cleaned up by the method of acid hydrolysis according to the standard protocol.

antibody, 55.8 ± 15.1% (n = 20) of total radioactivity was captured on glass (if incubation was overnight at 4 °C). The 2-h incubation at 4 °C of the assay mixture in the presence of the antibody reduced both the adsorption and the scatter of values to 25.4 ± 6.7%. On the glass overnight incubation resulted in further reduction of adsorption (17.3 ± 3.9%). On the basis of these results, polypropylene tubes were not used for evaporation from hexane, because PCB evaporates were minimally soluble in the mixtures mentioned above. The capture of radioactivity in polypropylene tubes was in all cases higher than 90%, including the ultrasound treatment in the process of solubilization.

Calibration and Selectivity: Characteristics of Antisera. The curves of the dose response for the three tested antisera were determined according to the standard RIA protocol. From the displacement curves in Figure 2 the different dose response to Delor 106 is evident. Antiserum GTG-1 exhibited a smaller curve slope of the dose response in comparison with antisera RTG-3 and GHC-1 in the concentration range 0.125–5 ng of Delor 106/tube. The spectrum of dose responses of antiserum GHC-1 to four Delor formulations is illustrated in Figure 3. From those curves it is evident that this antiserum is capable of detecting 0.1 ng of Delor 104, 105, and 106 in a tube with the total highest sensitivity to Delor 105. Thus, the antiserum will detect Delor 105 and 106 in the sample with higher sensitivity than Delor 104 but will exhibit low sensitivity to Delor 103 (see also Table I). As a calibration standard for the screening detection of PCBs in fat materials, we used animal fat with a defined concentration of 5.58 mg of Delor 106 kg^{-1}. The above antiserum showed approximately linear calibration dependences between the decrease in bound radioactivity and the increasing dose of Delor 106. Figure 4 shows a good stability of this calibration in curves obtained over the course of 8 weeks.

Detection selectivity is determined by the degree of specificity of the antibodies. The results of cross-reactions among Delor 106 and related formulations of PCBs or related chemical structures are presented in Table I for the three antisera. The values of cross-reactions were calculated according to the method of Abraham (1969). The more chlorinated formulations of Delor 106 and 105 showed—in accordance with immunochemical assumptions—an adequately higher cross-reactivity than the less chlorinated Delor 103. In all antisera the highest cross-reaction was found in 3,3′,4,4′,5-pentachlorobiphenyl, which is most similar to the conjugated hapten structure. The coplanar structure 3,3′,4,4′ showed a higher cross-reaction in two antisera when compared with the 3,3′,4,4′,5,5′ coplanar congener. A high degree of inhibition in RTG-3 and GTG-1 sera was demonstrated by the 2,2′,4,4′,5,5′ congener, which is one of the major components of technical PCB formulations (Johansson, 1989) occurring in human tissues (Newsome and Shields, 1981). Less similar to the homologue structure, 2,2′,4,5,5′-penta and 2,2′,3,3′,4,4′,5,5′-octa congeners exhibited a lower inhibition ability. In biphenyls with a lower number of chlorine atoms an adequately low cross-reaction was found. All antibodies were negligibly sensitive to DDE and practically did not react at all with DDT, TCDD, chlorinated benzenes, phenols, hexanes, polycyclic aromatic hydrocarbons, and steroids.

Precision. Intra-assay precision was established by measuring Delor 106 concentration in fat samples with different PCB contents. The routine procedure included the workup of extracted fat by acid hydrolysis method and the RIA detection. The recovery of Delor 106 from

Table I. Cross-Reactivity of Antisera against 3,3′,4,4′-Tetrachlorobiphenyl-6-azo–Protein Immunogens

substance (congener)	cross-reaction,[a] %, for antiserum RTG-3	GHC-1	GTG-1
Delor 106	100.0	100.0	100.0
Delor 105	160.9	183.3	177.0
Delor 104	47.7	51.9	52.3
Delor 103	15.8	17.3	
3,3′,4,4′-	250.0	80.0	50.0
3,3′,4,4′,5-	750.0	270.0	200.0
3,3′,4,4′,5,5′-	40.0	32.0	66.0
2,2′,4,4′,5,5′-	75.0	18.5	125.1
2,2′,3,3′,4,4′,5,5′-	12.0	5.0	3.5
2,2′,4,5,5′-	17.5	6.6	22.7
2,4,5-	9.0	1.5	0.4
4,4′-	4.0		
2-	≪0.01	≪0.01	≪0.01
DDT	<0.01	<0.01	<0.01
DDE	0.4		
TCDD	<5.0	<5.0	<5.0

[a] Cross-reaction defined as [(Delor 106 concentration for 50% displacement of tracer)/(concentration of structural analogue for 50% tracer displacement)] × 100.

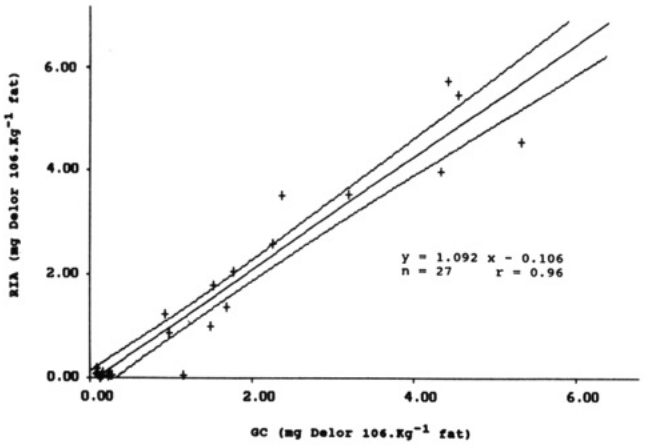

Figure 5. Comparison of PCB levels in cow milk measured by GC and RIA. The graph represents a regression line with 95% confidence band.

fortified fat for concentrations 0.97 and 5.0 ng of Delor 105 kg^{-1} of fat was about 75%. Intra-assay coefficients of variation (CV) in samples containing 0.5, 1.25, and 2.8 mg of Delor 106 kg^{-1} of fat were 21.2 (n = 40), 9.0 (n = 17), and 11.9% (n = 23), respectively. Interassay variation was calculated from values obtained in different assays carried out over the course of 2 months. The corresponding CV for samples with the average concentrations of 0.5 and 2.8 mg of Delor 106 kg^{-1} of fat were 23.9 (n = 37) and 24.6% (n = 30), respectively.

Comparison of RIA and GC. PCB detection in 27 extracts of milk purified on Florisil was carried out by GC in the Dairy Research Institute in Prague. The recovery of Delor 106 in this method was a minimum of 96%. The cleaned up evaporates of samples were reanalyzed in our laboratory by the RIA method using a Delor 106 standard. From the comparison of PCB levels it is evident that the radioimmunoassay yielded comparable values and reliable information about the level of contamination in 26 of the milk samples (Figure 5). In only one case was a great difference found which, however, could not be confirmed by a repeated analysis due to lack of material. We believe that the value discrepancy in one sample was caused by a mistake in the GC laboratory because the accuracy for negative RIA values approached 100% (n > 100 samples). The correlation of results obtained by these two methods is demonstrated in Figure 5 by the statistically significant coefficient r = 0.96 (n = 27). The results of PCB

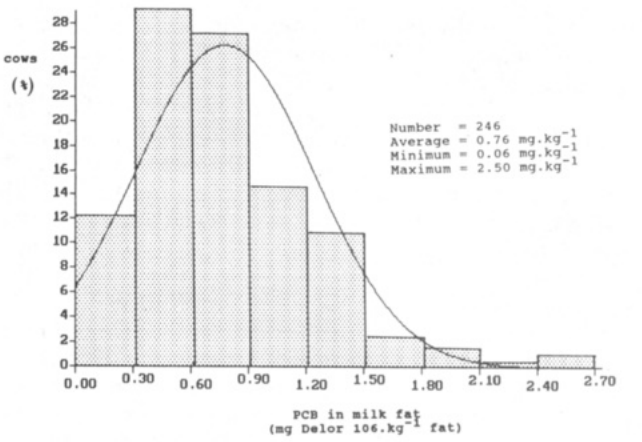

Figure 6. Distribution of PCB levels in the milk of cows of contaminated herd.

Table II. Comparison of PCB Residue Concentrations in Samples of Animal Fats Determined by RIA and GC

	mg of Delor 106 kg^{-1} of fat									
RIA	0.04	0.04	0.13	0.17	0.43	5.20	5.42	1.74	0.19	0.20
GC	0.03	0.03	0.16	0.20	0.28	5.48	4.10	2.60	0.15	0.17

Table III. PCB Levels before and after Elimination of Highly Contaminated Cows from Herd

method of determination	mg of Delor 106 kg^{-1} of fat: contaminated herd	after separation of cows
individual samples (mean value) RIA	0.76	
pool RIA	0.74	0.12
pool GC	0.70	0.14

determinations in different field samples of animal fats are summarized in Table II. The extracts cleaned up on Florisil were prepared and analyzed by gas chromatography by the inspection laboratory at Jihlava using the standard Aroclor 1260. The same extracts were reanalyzed after evaporation by RIA using the standard Delor 106. From the results listed in Table II good comparability of values obtained by these methods is evident in the concentration range 0.04–5.5 mg of Delor 106 kg^{-1} of fat.

Monitoring of Contaminated Cow Herd. PCB concentration in milk fat in 246 cows was measured by the presented Spheron microcolumn RIA technique. Figure 6 shows that this technique can become a useful tool for monitoring PCB distribution in a contaminated herd. In this experiment the milk fat was cleaned up by the method of acid hydrolysis. From individual analyses of milk samples the mean level of 0.76 mg of Delor 106 kg^{-1} of fat was calculated with the limit values of 0–2.5 mg kg^{-1} of fat. When the milk of all cows was mixed, a pool sample was obtained in which practically the same PCB level was found if the detection was carried out by the RIA or the GC method (Table III). The removal of the most contaminated cows from the herd resulted in an adequate lowering of PCB level in the pool reservoir.

DISCUSSION

In a series of earlier studies we demonstrated the fact that steroid haptens attached by a short azo bridge to the protein stimulate the formation of high-affinity antibodies with a broad range of cross-reactions among the homologous hapten and the related structures (Fránek and Hruška, 1980, 1983, 1985; Fránek et al., 1988). The immunoassays incorporating these antibodies offer a potential for the detection of a broad spectrum of structurally

related molecules retaining selectivity toward the given class of compounds. The connection of the biphenyl hapten with the protein carrier by means of the azo group results in the formation of antibodies that have an increased ability for the cross-reaction among tetra–heptachlorinated congeners and that do not react with chlorinated aromatic and cyclic hydrocarbons (Table I). In the classical concept of immunological specificity the close fit of the hapten determinant into the binding site represents a high degree of complementarity, since the regions complementary in shape are understood as being specific (Fránek, 1987). In our case the specific region to tetrachlorinated biphenyl includes about half of the hapten, whereas the remaining portion of the molecule lies outside the region of antibody complementarity. The competition for the binding site is thus entered not only by the highly active coplanar 3,3′,4,4′ congener but also by those biphenyls that have additional chlorine atoms situated on the opposite side to the homologous tetrachlorine moiety. These atoms, figuratively speaking, are not seen by the antibody, which enables the radioactive tracer to be displaced from the binding site by these competitors. The stereochemistry of the phenyl rings is an important factor in the competitive mechanism. Thus, the di-2,2′,4,4′,5,5′-hexachlorobiphenyl exhibits a high cross-reaction (18.5–125.1%), although the phenyl rings rotate in the noncoplanar orientation (McFarland and Clarke, 1989). It can be expected that the introduction of the bridge substituent into the biphenyl skeleton will more or less change the angle of rotation between the planes of the phenyl rings or form a steric hindrance of this rotation. The conformational change in an isomer thus formed can usually be more specifically fixed in monoclonal antibodies than in the polyclonal antiserum. A number of monoclonal antibodies with a defined immunochemical selectivity were prepared against 2,3,7,8-TCDD and used for the development of competitive ELISA for dioxins (Stanker et al., 1987). Highly toxic coplanar PCB congeners can become also attractive candidates for preparing selective monoclonal antibodies, since the difficulty of their detection by classical analysis is comparable with that of dioxins (Smith et al., 1990). The substitution of chlorine by the azo bridge in the meta or para position in the respective structures offers better molecular assumptions for coplanar conformations than the bridge introduced into the ortho position. In the ideal case monoclonal antibodies will possess stereospecificity toward coplanar 3,3′,4,4′, 3,3′,4,4′,5, and 3,3′,4,4′,5,5′ structures which will permit their recognition in the mixture of many other congeners and chlorinated hydrocarbons.

The development of immunoassay systems for chlorinated aromatic hydrocarbons is limited by the selection of solvents and working materials. The solvents must enable good solubility of the analyte in the reaction medium without undesirable effects on the conformation of the binding site of the antibody and must reduce its adsorption to the solid phase. The effectiveness of the solubilization of evaporates increases by ultrasonification. Stanker et al. (1987) obtained 50–60% of the radioactivity of ^{14}C-labeled dioxin in the BSA solution after a 2-h ultrasound treatment in glass tubes. Dioxin thus sonified was detected by competitive ELISA. The results obtained in this paper show that by the solubilization of Delor in 25% DMSO the yield without the use of ultrasound treatment was more than 80% of the original amount of PCBs. Newsome and Shields (1981) solubilized evaporates of Aroclor 1260 in 25% DMSO using ultrasound treatment and used conventional charcoal suspension in 25% DMSO for separation of RIA mixture. Aroclor 1260 was determined in human and cow milk with accuracy comparable to that with use of GC. When repeating this procedure, we found that the addition of a charcoal suspension into the reaction mixture in glass tubes resulted in a bad reproducibility of the calibration curves. The reason of this phenomenon is hard to explain. Nevertheless, these problems were removed by transferring the reaction mixtures (after incubation) from glass to polypropylene tubes, but this two-step procedure was rather tedious. Stable assay parameters were subsequently obtained by introducing modified Spheron microcolumn separation which we had originally developed for the radioimmunoassay of steroids (Fránek et al., 1988). The principle of this microcolumn separation is the adsorption of the free fraction of the reaction mixture to Spheron after it passes through the microcolumn. Unlike precipitation techniques using poly(ethylene glycol) or a second antibody, the microcolumn method maintained a stable low level of nonspecific binding, which resulted in a high sensitivity of detection. The method requires only a small amount of DMSO in the reaction mixture without the use of a centrifuge.

The choice of polymeric materials for laboratory work with PCBs requires circumspection to avoid errors in the respective procedure. As follows from the quantitative studies of Cseh et al. (1989), the use of hard plastic materials can only be recommended in cases if the contact time is short and losses in mass due to adsorption are negligible. Some plastic and practically all rubber materials including silicon, Norprene, and Tygon tubing in peristaltic pumps must be eliminated because of the danger of adsorption losses. The only safe material for work with PCBs is Teflon, for which no significant losses in mass have been found even after contact for many days. From our results it is evident that the use of plastic tips for routine pipetting of trace concentrations of PCBs has not resulted in any substantial losses in mass or in the variability of assay values. Intra-assay variation coefficients were about 10%. On the other hand, macroporous hydrophilic gel Spheron with the matrix consisting of a copolymer of hydroxyethyl methacrylate with ethylene dimethacrylate effectively adsorbed the lipophilic analyte, the amount of nonspecifically eluted radioactivity under conditions of microcolumn separation not exceeding 2%. The capture of the free fraction of PCBs was, as expected, more effective due to the small particle size of Sepheron (0.025–0.040 mm), which means an increase in its surface and subsequent slowing down of the flow rate. During the passage of the reaction mixture through the microcolumn, the PCB–antibody complex was reliably protected against capture on Spheron. It is evident that the adsorption interaction of polychlorinated aromates with laboratory materials can be the cause of developmental problems, if the immunochemical detection is to be carried out on, say, microtitration plates. Adsorption effects observed on solid phase can overlap with little solubility of lipophilic substances in the reaction medium. It is difficult to decide which of these effects prevails. Kennel et al. (1986) tested chlorinated dioxins on microtitration plates by indirect ELISA. Immobilized hapten bound the monoclonal antibodies but did not compete with 2,3,7,8-TCDD solubilized in detergent. That might have been—according to the authors—due to low solubility of dioxin in detergent or to unsuitable structural relations in this hapten–antibody interaction. Similar problems were established in our laboratory in the effort of replacing the radioimmunoassay by ELISA with immobilized 3,3′,4,4′-tetrachlorobiphenyl–BSA. Delor 106 added to the wells of the plate did not compete with immobilized hapten for binding on the antibody in the medium containing

25% DMSO in the assay buffer. Also, in this case the cause of this noncompetition can be due to too high an affinity of the antibody to immobilized hapten (the phenomenon of the bridge binding) or because of the loss of mass through adsorption on polystyrene.

Since the late 1970s only two papers have been published on the development of RIA PCBs (Luster et al., 1979; Newsome and Shields, 1981). According to our knowledge these methods have not been introduced into practice on a large scale. The application of the microcolumn RIA for screening of a large number of milk samples showed that RIA was a cheaper and quicker detection means than chromatographic methods. Promising prospects of future utilization consist of simplification of the immunodetection system, by increasing the congener selectivity with the utilization of monoclonal antibodies and by replacing ^{125}I-labeled haptens by nonisotopic markers. Nonseparation techniques, such as polarization fluoroimmunoassay (PFIA), offer many advantages in this respect, despite a lower detection sensitivity as compared with that of RIA or ELISA. It can be noted that the extraction and concentration of the analyte from samples prior to analysis essentially avoid this problem.

ABBREVIATIONS USED

GC, gas chromatography; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; RIA, radioimmunoassay; NMR, nuclear magnetic resonance; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; TG, porcine thyroglobulin; KLH, keyhole limpet hemocyanin; DMSO, dimethyl sulfoxide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ELISA, enzyme-linked immunosorbent assay.

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