ISOLATING LARGE AMOUNTS OF BIOACCUMULATED PERSISTENT ORGANIC POLLUTANTS (POPs) FOR TOXICITY TESTS

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Introduction

A direct measure of the toxicity of persistent organic pollutants (POPs) is to use the exact bioaccumulated mixture to determine various biological effects. To obtain sufficient quantities of the contaminant mixture for extensive dose-response toxicity testing, large amounts of tissue (1-50 kilograms) must be extracted and purified. This purified extract is transferred into a small volume of non-toxic lipophilic vehicle amenable to the bioassay. We have developed a method for the large-scale isolation and analysis of POPs including polychlorinated dibenzodioxins and furans (PCDD/PCDFs), polychlorinated biphenyls (PCBs), non-ortho PCBs, polyaromatic hydrocarbons (PAHs) and organochlorine pesticides (OCPs). This approach offers a facile and accurate means of isolating large amounts of bioaccumulated POPs for toxicity testing.

The first steps involve extraction of the tissue followed by cleanup of bulk lipids by dialysis through a polyethylene membrane. Then, depending on the classes of POPs targeted, the procedure involves either destructive or non-destructive methods to eliminate co-extractants that interfere with the final volume reduction and the bioassay. The last step is accurate formulation of the dosing solution in a metabolically neutral vehicle such as triolein. The method incorporates steps for circumventing problems in preparing dosing solutions with viscous vehicles. The efficiency of the large-scale isolation process is monitored by comparing levels of POPs in the purified extract to levels in the fish tissue. The final product is a well-characterized dosing solution amenable to fish egg and bird egg embryotoxicity (egg injection) studies [1,2].

Methods and Materials

Kilograms (1-50) of fish, avian eggs, or other tissue samples are stored frozen until initiation of the procedure. Individual portions of tissue are ground, then combined, and the resulting composites are ground to produce a homogeneous mixture. Aliquants of the mixture are set aside for determination of POPs concentrations and percent lipid. The remaining material is dried with four times its weight of anhydrous sodium sulfate then distributed between several large glass columns (6 cm i.d. x 80 cm, with 1L reservoirs) that hold 400g of the tissue and attendant sodium sulfate. Each column is extracted with 1.8L of dichloromethane (Fisher Optima grade or the equivalent). The solvent is rotoevaporated and common extracts are pooled. The contaminants are then separated from the kilogram quantities of lipids by dialysis [3].

The dialytic cleanup involves loading 50mL portions of the extract into contaminant-free layflat low density polyethylene membranes (PE) tubes. The PE is 5 cm wide and has a 50 micrometer wall thickness (Brentwood Plastics, Inc. St. Louis, MO, USA). The tubing is heat-sealed and then submerged in a glass jar containing 1800mL of 20% dichloromethane/80% hexane. The contaminants are dialyzed for two 3-4 day periods. This time period is determined by monitoring dialysis of ^{14}C-2,2',5,5'-tetrachlorobiphenyl and ^{14}C-mirex. The tetrachlorobiphenyl effectively

tracks recovery of PCBs. The mirex is suitably large and lipophilic to represent those compounds that are near the size limit posed by the theoretical transport corridors of the polyethylene membrane. The extracts are pooled and the amount of lipid in the dialysate is determined gravimetrically.

Residual lipids/biogenics that can interfere with the bioassay are moved at this stage. Also, class specific fractionations are performed at this time. Removal of PAHs may be necessary, as they interfere with the dioxin-like toxicity assessment [4]. PAHs and residual lipids are removed by reactive cleanup with sulfuric acid impregnated silica gel. If a full range of POPs is targeted for toxicity studies, the lipids are removed using a glass column packed with 20 g SX3 Biobeads (Biorad Laboratories, Richmond CA) and 80/20 hexane/dichloromethane elution, followed by HPGPC (Phenogel, Phenomenex, Torrance CA) with methylene chloride. An alternative clean up of problematic lipids and cholesterol is the cold filtration method. In this procedure the extract is cooled to precipitate the lipids and cholesterol which are then filtered [5]. After clean up, solvent volume is reduced and the extract is transferred to a test tube.

The dose needed for the toxicity test is determined, and then portions of the extract are quantitatively exchanged into triolein, using the following procedure. A sufficient amount of triolein is filtered through a 0.22 micron filter to remove extraneous particles and most bacteria. Positive displacement measuring devices are used for all steps. Because of the high viscosity of triolein, it is diluted: a 50/50 triolein/solvent mixture is prepared by pouring triolein into the barrel of a syringe and then dispensing an accurate volume into a test tube; an equal volume of a solvent such as pentane or dichloromethane is added to the test tube. At this point, a vial containing a portion of purified tissue extract is reduced to near dryness under a gentle stream of nitrogen. The diluted triolein is added at twice the final desired volume of the dosing solution. The vial is capped and sonicated for 3 minutes. The solvent is then evaporated under a stream of nitrogen until little volume change is noted. The vial is then placed in a heating block and the blow-down is continued until no further volume change is evident. The headspace in the vial is filled with argon or nitrogen and then capped.

Contaminant concentrations are determined in the original tissue and in the final extract prepared for the dosing solution. Targeted compounds include PCDD/PCDFs, PCB congeners, non-ortho PCBs and OCPs [6, 7, 8]. The method for analysis of PCB congeners and PCDD/PCDFs includes reactive cleanup, HPGPC, fractionation by high pressure porous graphitic carbon chromatography followed by pressurized alumina chromatography. Gas chromatographic (GC) analysis for congener-specific PCBs is performed on a Hewlett-Packard model 5890 Series II. Analysis of PCDD/PCDFs and non-ortho PCBs is performed by GC/MS on a VG-70S High Resolution MS.

Results and Discussion

This procedure allows large amounts of bioaccumulated contaminants to be isolated from very large masses of tissue and prepared for toxicity testing while maintaining the integrity of the contaminant mixture. The quality control accompanying the procedure assures an accurate solution is prepared--an important consideration in producing test mixtures.

Several factors have been recently identified that improve the accuracy of the isolation procedure:

1. Before the large-scale dialysis is conducted, it is critical to pre-extract the polyethylene to remove absorbed airborne contaminants, especially PCBs as they are prevalent in indoor air of older buildings that contain fluorescent lamps. The polyethylene wash is analyzed to verify lack of contaminants in the polyethylene.

2. Careful monitoring of the dialytic recoveries with 14-C labeled mirex spiked in surrogate oil enables optimization of dialytic recoveries for various thickness of polyethylene.
3. The reactive cleanup procedure for removal of PAHs destroys certain OCPs, such as *heptachlorepoxide, dieldrin, and methoxychlor* [9]. *It is advisable to use gravity-flow* GPC, followed by HPGPC when isolating these and other susceptible compounds.
4. Cold filtration can be used to remove problematic lipids. The filtered lipids are analyzed to quantify any potential losses of compounds of interest.
5. It is advisable to prepare the final solution just prior to the toxicity test. Final solutions should be stored at room temperature to assure that all compounds remain soluble.
6. The major obstacle to accurate preparation of the dosing solution is the injection vehicle's viscosity. This problem is minimized by dilution with a volatile solvent such as dichloromethane, and exclusive use of positive-displacement measuring devices.
7. The use of the heating block to evaporate the solvent does not cause selective losses of compounds *via volatilization from the triolein.*

Using these latest advances in our procedure for isolating contaminants for bioassay testing, we have recently isolated POPs from fish obtained from a river that is highly contaminated with a complex mixture of PCBs, PCDD/PCDFs and OCPs. A large mass of fish (15kg) was obtained from each of 3 contaminated sites and from a control site. The four 15kg portions contained a total of 3000 grams of lipids from which the POPs were isolated and dosing solutions prepared. Analytical verification of the isolation procedure was conducted. Dosing solutions were prepared and injected into fish eggs to study embryotoxicity.

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