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Short Communication

HPLC DETERMINATION OF TOCOPHEROL, RETINOL, DEHYDRORETINOL AND RETINYL PALMITATE IN TISSUES OF LAKE CHAR (*SALVELINUS NAMAYCUSH*) EXPOSED TO COPLANAR 3,3′,4,4′,5-PENTACHLOROBIPHENYL

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Abstract—Tocopherol, retinol, dehydroretinol, and retinyl palmitate were measured by reversed-phase HPLC in liver, kidney, and plasma of lake char exposed to orally administered coplanar 3,3′,4,4′,5-pentachlorobiphenyl (PCB). Tocopherol concentrations were unaffected after eight weeks. Liver retinol, dehydroretinol, and retinyl palmitate concentrations were lower, whereas kidney retinyl palmitate was elevated in PCB-exposed groups. Tissue retinoid concentrations provide sensitive indicators of coplanar PCB exposure in fish.

Keywords—Vitamin E Vitamin A Retinoids Coplanar PCB

INTRODUCTION

A variety of important physiological processes depend on adequate levels of tocopherol (vitamin E) and retinol (vitamin A). Tocopherol is important because of its activity as an antioxidant in protecting cellular and subcellular membranes [1]. Retinol deficiency in fish is associated with loss of vision, edema, depigmentation, and impaired growth [2]. Both are essential dietary elements and have received increasing attention as indicators of exposure to a variety of environmental contaminants. Chemicals that bind with Ah receptor induce biotransformation enzyme activities that in turn can increase oxidative stress [3], leading to a reduction of tissue stores of tocopherol [4]. Vitamin A concentrations in kidney, liver, and serum of rats are altered by exposure to organic pollutants, particularly chemicals (e.g., coplanar PCBs) that interact with the Ah receptor [5].

Fish liver and liver oils represent rich sources of retinoids and tocopherol [6]. Retinol and retinyl palmitate have been identified as bioindicators of exposure to organic contaminants in mammals and birds [5,7,8]. However, information about retinoid stores in fishes exposed to organic contaminants is limited [9]. Recently, Arnaud et al. [10] described a rapid, effective reversed-phase HPLC method for measuring both retinol and tocopherol. Although this method was developed for mammalian serum samples, we present results showing its utility for quantifying tocopherol, retinol, dehydroretinol, and retinyl palmitate in liver, kidney, and plasma of lake char (*Salvelinus namaycush*). Moreover, similar methodology was recently used to quantify retinoids in serum, eyes, and eggs of rainbow trout (*Oncorhynchus mykiss*) [11]. We also present preliminary data examining the effect of a single oral dose of 3,3′,4,4′,5-pentachlorobiphenyl (PCB congener 126) on tocopherol, retinol, dehydroretinol, and retinyl palmitate in liver, kidney, and plasma of lake char eight weeks after ingestion. In this study, the PCB doses produced muscle concentrations similar to those previously reported in fish from the Great Lakes and other freshwater systems [12,13].

MATERIALS AND METHODS

Juvenile lake char, obtained as eggs from Whiteswan Lake, Saskatchewan, (247 ± 44 g) were acclimated for four weeks in 140-L Fiberglas® tanks receiving at least two L/g fish per day of dechlorinated Winnipeg city tap water (12 ± 2°C, $CaCO_3$ 82.5 mg/L, pH 7.8). Fish were held on a 12:12-h light:dark photoperiod and maintained with a diet of commercial Martin Feed Mills pellets (Elmira, Ontario) at 1% of body weight every second day. [^{14}C]PCB 126 (specific activity 8.7×10^{11} Bq/mol) and all other reagents and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The PCB was dissolved in ethanol and warmed gelatin [14]. Fish were treated with a single oral dose of gelatin/ethanol at 2 ml/kg, containing nominal concentrations of 0 (control), 1.2 (low dose), or 40 μg (high dose) [^{14}C]PCB 126 per kilogram fish weight. Capillary GC analysis of the PCB 126 showed a purity of >99.5%. Extractable radioactivity was determined after eight weeks by homogenizing freeze-dried muscle in toluene and assaying portions of this extract by liquid scintillation counting. Following gavage, fish were returned to the same tanks.

After eight weeks, fish were anesthetized for 5 min with tricaine methanesulphonate neutralized to tank pH with ammonium hydroxide. Blood was collected from the caudal vessels with preheparinized 3-ml syringes and immediately centrifuged to obtain plasma. Tissues were dissected and put into sterile plastic bags, and immediately frozen with the plasma at −120°C until analyzed. We used distilled deion-

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ized (Millipore®, Milli Q, Bedford, MA) water as a homogenizing medium for tissues. To precipitate proteins after homogenization, 200 μl HPLC-grade ethanol was added to 200 μl tissue homogenate or plasma. Plasma, liver, and kidney samples were extracted with 500 μl (3:2, v/v) ethyl acetate/hexane. Residues from the ethyl acetate/hexane extracts were redissolved in mobile phase and injected (20 μl) onto a 3-μm-bead-size Adsorbosphere HS C_{18} column (4.6 mm i.d., 150 mm length) with attached 10-mm Adsorbosphere guard column (Alltech Associates, Deerfield, IL).

The HPLC system consisted of two model 302 solvent pumps, a model 231 sample injector, a model 704 system controller, a model 620 data module, and a model 116 dual channel UV absorbance detector. The detector was set at 292 nm for tocopherol and tocopherol acetate detection, and 325 nm for retinol and retinyl palmitate detection (Gilson Medical Electronics, Milwaukee, WI). The column was thermostated to 26°C, and samples and standards were eluted isocratically with acetonitrile:methanol:water (70:20:10, v/v/v) delivered at a flow rate of 1.0 ml/min. Standard retinol, retinyl palmitate, tocopherol, and tocopherol acetate were purchased from Sigma Chemical Co. (St. Louis, Mo.), and 3,4-dehydroretinol was a gift from H. Keller (F. Hoffmann-La Roche Ltd., Basel, Switzerland). As with previous work [15], there was no loss of these compounds after tissue storage at −110 ± 1°C for 30 d.

Reproducibility of the method was evaluated by 10 measurements of tocopherol and retinoids in the same tissue. Tocopherol acetate was used as an internal standard, with the recovery of a known spike (575 ng) used to correct for the efficiency of each extraction. Recovery efficiencies were determined by spiking known amounts of tocopherol (875–4,375 ng) and retinoids (8–400 ng). Varying amounts of tissue (2.5–50 mg) or plasma (50–200 μl) were analyzed to examine the correlation between the amount of tissue and the concentration of tocopherol and retinoids.

Data were analyzed using ANOVA. Pairwise comparisons were conducted with Fisher's LSD and Duncan's tests. A probability level of 0.05 was considered significant.

RESULTS AND DISCUSSION

Extracted standards eluted with characteristic retention times (Fig. 1A) that did not differ between preparations. Figure 1 also shows typical elution profiles from extracted lake char liver (Fig. 1B), kidney (Fig. 1C), and plasma (Fig. 1D). Although both liver and kidney contained substantial amounts of tocopherol, retinol, dehydroretinol, and retinyl palmitate, levels of retinyl palmitate and dehydroretinol were not routinely detectable in plasma.

A dilution series of liver tissue ranging from 2.5 to 50 mg tissue in 200 μl homogenate showed linearity of detection for tocopherol, retinol, dehydroretinol, and retinyl palmitate with r^2 values of 0.997, 0.997, 0.996, and 0.933, respectively. Similar results were obtained when 50- to 200-μl volumes of plasma were used. Routinely, 10 mg of both liver and kidney in 200 μl homogenate or 200 μl plasma was extracted, which allowed reproducible results while using the least practical amount of tissue. When tissue or plasma samples were spiked with known amounts of tocopherol or retinoids, recovery of the spikes was 93.0 to 94.8% and 87.7 to 98.5%, respectively. Tocopherol acetate, used as an internal standard, corrected for differences in sample extraction efficiencies for liver (77.3 ± 3.9%), kidney (75.1 ± 4.5%), and plasma (68.4 ± 1.0%). The precision of the method was evaluated by repeated measures of a single tissue sample. Ten separate extractions of 10 mg tissue led to SE measures of 1.14% for tocopherol and 2.18 to 4.21% for the retinoids, respectively. Similar results (2.4–5.1%) were obtained with other tissue amounts (2–100 mg).

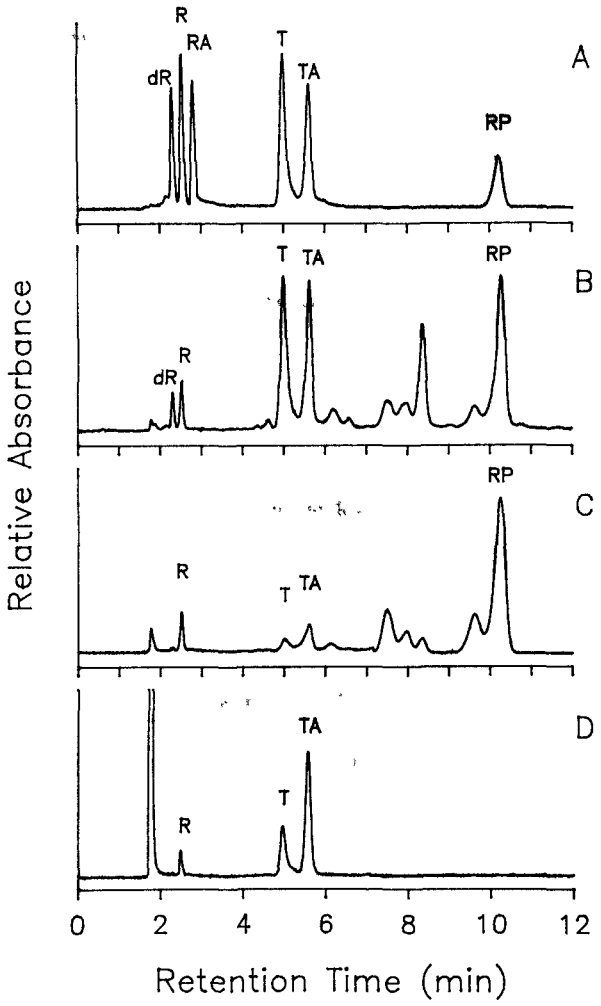

Fig. 1. HPLC chromatograms for (**A**) a standard containing 185 ng dehydroretinol (dR), 80 ng retinol (R), 80 ng retinol acetate (RA), 875 ng tocopherol (T), 570 ng tocopherol acetate (TA), and 400 ng retinyl palmitate (RP); (**B**) liver; (**C**) kidney; and (**D**) plasma of lake char. The detector was set at 325 nm from 0 to 4 min, 292 nm from 4.01 to 7 min, and 325 nm from 7.01 to 12 min for each chromatogram. Full-scale deflection for chromatograms A, B, C, and D were 95, 43.3, 664.2, and 674.6 mV, respectively.

Eight weeks after the oral dose, muscle PCB 126 concentrations of 0.622 ± 0.136 and 12.69 ± 0.896 μg/kg were found in the low- and high-dose groups, respectively. These concentrations are environmentally relevant because similar concentrations of Ah-inducing chemicals have been reported in Great Lakes fish [12] and in fish from marine systems [13].

Concentrations of tocopherol in liver, kidney, and plasma were similar to those we previously reported in rainbow trout using a different analysis technique [16] and to those reported in other fish [17,18]. Moreover, retinol, dehydroretinol, and retinyl palmitate concentrations were near concentrations previously reported in fish [9,11,19].

There were no differences between treatments in tocopherol concentrations in plasma or liver. In kidney, fish exposed to the low PCB dose showed a slight but significantly higher tocopherol concentration than control or high-dosed groups (Fig. 2A). These results are surprising, given that previous work has shown an increase of oxidative stress in rats exposed to Ah-inducing compounds similar to PCB 126 [20] and that tocopherol stores can decline when exposed to oxidative radicals [21]. Exposure to PCB 126 for eight weeks may be insufficient to produce oxidative stress capable of depleting tocopherol stores in lake char.

Retinol and dehydroretinol concentrations in liver of PCB-exposed fish were lower than levels in livers of fish treated with gelatin containing no PCB (Fig. 2 B, C). Moreover, the high-dose fish had more severely depressed retinol than the low-dose group. Although the high-dose group appeared to have higher kidney retinol stores, there were no significant differences in either retinol or dehydroretinol concentrations. In plasma, the high-dose group had lower plasma retinol than control and low-dose fish.

The data from the high-dose group of fish in this study (Fig. 2C) appear to support recent mammalian studies [22,23] showing that lower retinoid concentrations in the liver are concurrent with a decline of plasma retinol but higher kidney retinyl palmitate. High retinoids in the kidney may be the result of altered esterifying enzymes and represent an attempt to limit vitamin A loss [23] and minimize potential vision, growth, and metabolic disturbances.

There is growing evidence that liver retinol stores decline with exposure to dioxin-like contaminants in mammals [5,23,24] and birds [25]. Lower liver retinol stores are often associated with contaminants that bind to the Ah receptor

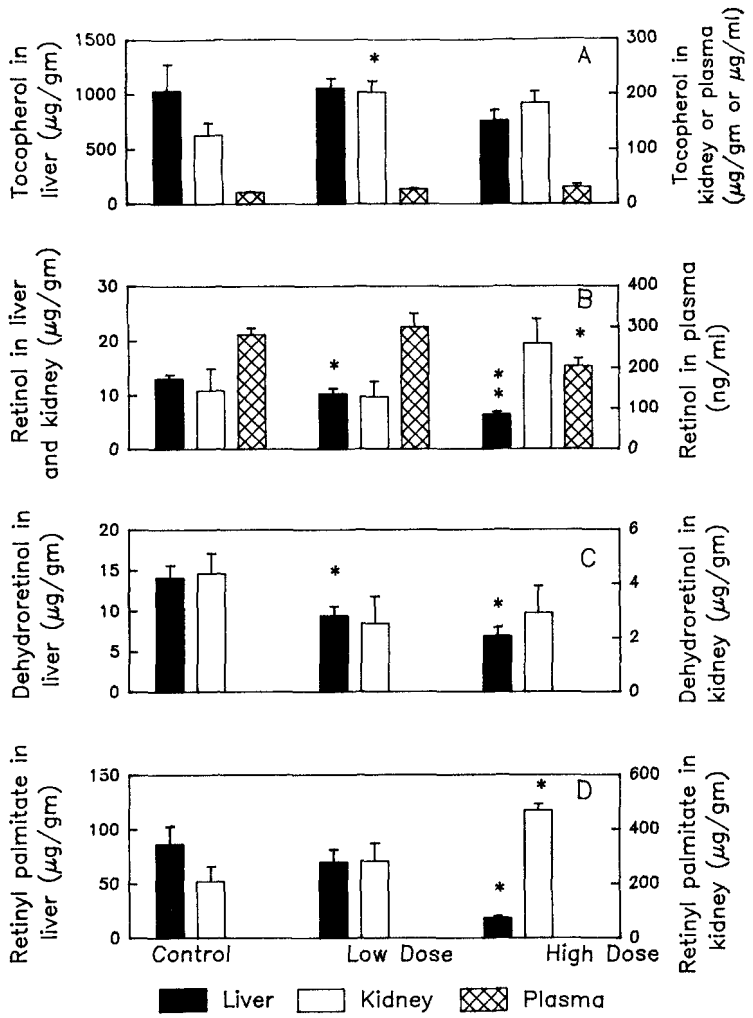

Fig. 2. **(A)** Tocopherol in liver, kidney, and plasma; **(B)** retinol in liver, kidney, and plasma; **(C)** dehydroretinol in liver and kidney; and **(D)** retinyl palmitate in liver and kidney of lake char exposed to 0 (control), 1.2 (low dose), or 40 μg (high dose) PCB 126 per kilogram fish weight. Asterisks denote statistical differences ($n = 5$, $p = 0.05$).

[5] and induce MFO enzyme activity. Induction of the phase 1 MFO enzyme activities and the phase 2 conjugating enzyme UDP-glucuronyltransferase is thought to account directly for increased metabolism of retinol in the liver [26,27]. Conversely, Brouwer and van den Berg [28] reported altered retinoid concentrations in mice exposed to PCB doses that did not induce MFO activity. They suggest that metabolites of PCB molecules interfere with the binding of transthyretin to retinol-binding protein (RBP). Unbound RBP may be filtered through the glomerular membrane, causing a loss of RBP and retinol. Because the liver can rapidly convert retinyl palmitate to retinol [28], these mechanisms may explain the concurrent decline of retinyl palmitate observed in lake char liver in this study.

We have shown that the method of Arnaud et al. [10] for simultaneously measuring tocopherol and retinol can effectively quantify tocopherol, retinol dehydroretinol, and retinyl palmitate in plasma, liver, and kidney tissues of freshwater fish. Although their dynamic mechanisms require examination, retinoids are promising bioindicators of exposure to PCB congener 126.

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