POLYCHLORINATED BIPHENYLS PATTERN ANALYSIS: POTENTIAL NONDESTRUCTIVE BIOMARKER IN VERTEBRATES FOR EXPOSURE TO CYTOCHROME P450-INDUCING ORGANOCHLORINES

NICO W. VAN DEN BRINK, ELZE M. DE RUITER-DIJKMAN, SIM BROEKHUIZEN, PETER J.H. REIJNDERS, and ALBERTUS T.C. BOSVELD

Institute for Forestry and Nature Research (IBN-DLO), Box 23, NL-6700AA Wageningen, The Netherlands

(Received 24 February 1999; Accepted 9 July 1999)

Abstract—Biomarkers are valuable instruments to assess the risks from exposure of organisms to organochlorines. In general, however, these biomarkers are either destructive to the animal of interest or extremely difficult to obtain otherwise. In this paper, we present a nondestructive biomarker for exposure to cytochrome P450-inducing organochlorines. This marker is based on a pattern analysis of metabolizable and nonmetabolizable polychlorinated biphenyl (PCB) congeners, which occur in several kinds of tissues (and even blood) that can be obtained without serious effects on the organism involved. The fraction of metabolizable PCB congeners is negatively correlated with exposure to PCBs, which are known to induce specific P450 isoenzymes. This relation can be modeled by a logistic curve, which can be used to define critical levels of exposure. In addition, this method creates an opportunity to analyze biomarker responses in archived tissues stored at standard freezing temperatures (∼20°C), at which responses to established biomarkers deteriorate. Furthermore, this method facilitates attribution of the enzyme induction to certain classes of compounds.

Keywords—Polychlorinated biphenyl pattern Nondestructive Biomarker Cytochrome P450

INTRODUCTION

Organochlorine pollutants have been of environmental concern for the last three decades. They have been detected in almost all compartments of the global ecosystem, and they can induce adverse effects in organisms. Critical assessment of the risks posed by the different compounds, either alone or in combination, to ecosystems is needed for the management of these systems.

Biomarkers have been proposed as tools for assessing such risks [1]. A biomarker can be defined as a xenobiologically induced variation in cellular or biochemical components or processes, structures, or functions that are measurable in a biologic system or in samples [2]. For the exposure of organisms to organochlorine compounds that induce cytochrome P450 (CYP) isoenzymes, alkoxyresorufin-O-dealkylase biomarkers have been developed and validated [3,4]. Of this group of biomarkers, 7-ethoxyresorufin-O-deethylase, which is associated with the activity of CYP1A1 [5], has been related to exposure to dioxinlike compounds with a significant affection for the Ah-receptor [4]. Moreover, ethoxyresorufin-O-deethylase induction has been associated with toxic effects, including effects on growth and reproduction [6]. The activity of mammalian CYP1A2 can be measured by methoxyresorufin-O-demethylase [7,8] and is also related to exposure to dioxins (3-methylcholanthrene–induction type). The activity of CYP2B1/2 can be measured using either pentoxyresorufin-O-depentylase or benzyloxyresorufin-O-debenzyllase [8,9] and is related to exposure to the phenobarbital type of inducers.

A major drawback of these enzyme activity-based biomarkers, however, is that they can only be analyzed ex vivo using freshly isolated tissue. Progressing ethical standards de-
further details, see [18]). For this study, whole specimens were analyzed.}

Pine martens, which were found dead at the Veluwe, a forested area in the center of the Netherlands, were dissected, and the subcutaneous fat was collected for PCB analyses. Five specimens of the white-toothed shrew, which is a prey item of the pine marten, were collected in 1993 at the floodplains of the river Waal at Geldersche Poort in the Netherlands, near the German border. Of these specimens, the liver was analyzed for PCBs. All shrews were adults, including two males and three females. Badgers, which were killed in road accidents, were dissected and their subcutaneous fat collected. The specimens were found at different sites in the Netherlands. (For further details see [19].) Earthworms, which are a major food item of badgers, were also collected in Geldersche Poort in 1993, in combination with the white-toothed shrew. Whole-body tissue was used to analyze PCBs.

Of 15 breeding Antarctic Adélie penguins from Hop Island, blood samples were collected at egg-laying, hatching, and later in the season, when the chicks formed crèches. This resulted in 45 samples being collected from a vein in the tarsus. (For details on sampling methods, see [20].)

**PCB analyses**

Liver samples were extracted in n-hexane for 6 to 8 h with soxhlet extraction after dehydration with anhydrous sodium sulfate. Fat samples were extracted in n-hexane under reflux. Worms were extracted by shaking in acetone-petroleum ether (1:1, v/v). Blood samples were stored frozen in a solution of sodium citrate and sodium chloride. After the addition of acetone, blood samples were extracted with hexane. (For details on preparation of blood samples, see [20].)

After extraction, all extracts were cleaned first with sulfuric acid in water (1:1, v/v) and then with column chromatography over silica and aluminum oxide. The clean extract was analyzed for individual PCB congeners using capillary gas chromatography with electron capture detection by a 63Ni detector. (For details on separation and detection, see [20].) As a definition, \( \Sigma \text{-PCB} \) is the sum of the PCB congeners 31, 28, 52, 61, 66, 95, 101/90, 151, 107/108, 149, 118, 146, 153, 132, 105, 141, 179, 138/164/163, 182/187, 183, 128, 174, 177, 180, 170, 196, 194, and 206 (IUPAC numbers according to [21]).

With incomplete separation, the peak was assumed to be the PCB congener with the first number (e.g., the peak most likely containing both congeners 101/90 is quantified as 101). The amount of fat was determined gravimetrically except in the case of blood samples, in which the fat content was determined fluorometrically [20]. In this study, \( \Sigma \text{-PCB} \) concentrations were expressed on a fat-weight (g) basis (e.g., \( \mu \text{g/g} \)). Quality of the chemical analyses was assured by participation in an interlaboratory calibration study organized by the International Council for the Exploration of the Sea, with adequate results [22].

**Statistics**

To obtain a normal distribution and homoscedasticity of the distribution, \( \Sigma \text{-PCB} \) concentrations were transformed into their natural logarithm before statistical analyses were performed. Differences between species were analyzed with Student’s \( t \)-tests [23]. The level of significance for differences was set at \( p < 0.05 \).

For an integrated overview of the data, a principal component analysis (PCA) was used [24]. This technique allows visualization of the patterns composed of the specific metabolizable and nonmetabolizable PCB congeners in the various species. The PCA was performed on the individual PCB congener concentrations relative to PCB153. Differences in PCB patterns between species were examined with a redundancy analysis using Monte Carlo Permutation tests [23]. The number of permutations was set at 200 and the overall significance at \( p < 0.05 \). Data concerning concentrations relative to PCB153 were not transformed before \( t \)-tests.

In the dose-response curves, the response parameter was defined as the sum of the concentrations of all metabolizable PCB congeners (IUPAC numbers: 31, 28, 52, 61, 66, 95, 101/90, 105, 141, 179, and 174) relative to \( \Sigma \text{-PCB} \). The curves were calculated using Genstat® 5.3 software (Lawes Agricultural Trust, Rothamsted, UK) [25]. Sigmoid curves were fitted to the data by maximum likelihood. Data on the metabolic fraction were assumed to have a Poisson distribution.

**RESULTS**

Results of the prey-predator couples are discussed first, followed by results of the pattern analysis based on the blood samples.

**PCB concentrations**

The PCB concentrations in the species examined were highest in the harbor seal, which is a marine predator (geometric mean, 29.9 \( \mu \text{g/g} \); Table 1). The herring, which is considered to reflect the prey items of harbor seals, contained significantly lower \( \Sigma \text{-PCB} \) concentrations (geometric mean, 1.3 \( \mu \text{g/g} \); Table 1). The pine marten and badger also showed significantly lower \( \Sigma \text{-PCB} \) concentrations than the harbor seal, at 5.3 and 2.6 \( \mu \text{g/g} \), respectively (\( t \)-test, \( p \ll 0.05 \), Table 1). The \( \Sigma \text{-PCB} \) concentrations in the white-toothed shrew and marten were com-

<table>
<thead>
<tr>
<th>PCB concentrations</th>
<th>Herring (6)</th>
<th>Harbor seal (87)</th>
<th>Wt. shrew (5)</th>
<th>Pine marten (19)</th>
<th>Earthworm (6)</th>
<th>Badger (12)</th>
<th>A. penguin (45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geomean (( \mu \text{g/g fat} ))</td>
<td>1.3</td>
<td>29.9</td>
<td>14.5</td>
<td>5.3</td>
<td>5.2</td>
<td>2.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Range</td>
<td>0.5–3.8</td>
<td>2.5–148.0</td>
<td>10.5–20.9</td>
<td>1.1–59.8</td>
<td>3.3–8.3</td>
<td>0.5–43.1</td>
<td>0.1–5.3</td>
</tr>
<tr>
<td><strong>Metabolizable fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average (% ( \Sigma \text{-PCB} ))</td>
<td>45</td>
<td>12</td>
<td>34</td>
<td>9</td>
<td>45</td>
<td>13</td>
<td>57</td>
</tr>
<tr>
<td>Rel. Stdev</td>
<td>15</td>
<td>73</td>
<td>20</td>
<td>78</td>
<td>5</td>
<td>98</td>
<td>17</td>
</tr>
<tr>
<td>Difference ( \Sigma \text{-PCB} )</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difference ( MF_{\text{PCB}} ) (%)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. \( \Sigma \text{-PCB} \) concentrations (\( \mu \text{g/g lipid wt} \)) and concentrations of metabolizable PCB congeners relative to \( \Sigma \text{-PCB} \) concentrations (\( MF_{\text{PCB}} \)%) in tissues of predators and their primary prey.
Fig. 1. Ordination diagram (principal component analysis) based on the relative concentration of the different polychlorinated biphenyl (PCB) congeners to PCB153 in several animal species. Metabolizable PCB congeners are indicated by open circles and nonmetabolizable by filled circles. Individual PCB congeners close to the symbol of a species are relatively overabundant in the PCB pattern of that species. The eigenvalue of the x and y axes are 0.83 and 0.08, respectively. This indicates that the x axis is more responsible than the y axis for variance in the data set.

parable (t-test, p > 0.05, Table I), as were those in the earthworm and badger.

**PCB congener pattern**

Figure 1 shows the output of a PCA based on the pattern of individual PCB congeners in the different prey and predator species (concentrations relative to PCB153). The earthworm samples are relatively dominated by metabolizable PCB congeners. In contrast, samples from predators are dominated by PCB153 and other nonmetabolizable congeners. The white-toothed shrew and herring occupy an intermediate position, indicating that neither metabolizable nor nonmetabolizable congeners dominate the PCB patterns in these species. The composition of the PCB mixtures in the three predator species and in their respective prey species are significantly different, as observed in a redundancy analysis and Monte Carlo Permutation tests (nperm = 200, p < 0.005).

The metabolizable fraction of Σ-PCB (MF_{pcb}, or the sum of all metabolizable PCB congeners relative to Σ-PCB) in samples from the harbor seal, pine marten, and badger ranged from 9 to 13% of Σ-PCB. In the earthworm and herring samples, the MF_{pcb} was, on average, 45%, whereas for the white-toothed shrew samples, the MF_{pcb} was, on average, 34% (Table 1). The MF_{pcb} had a relatively large range in the harbor seal, pine marten, and badger, whereas in the prey species, this range was smaller. When comparing predators and prey, the metabolizable fraction was always significantly lower in predators (t-test, p < 0.001; Table 1).

**Relation between PCB concentration and metabolizable fraction**

When the metabolizable fraction of Σ-PCB in the harbor seal was related to Σ-PCB, a significant relationship was found (Fig. 2). To model the MF_{pcb} at an exposure near 0 μg/g, without associated enzymatic metabolism of PCBs, the MF_{pcb} of the herring was incorporated. This situation was assumed so that the upper limit of the possible occurring MF_{pcb} in harbor seals could be assessed. For the calculation of the curve, the (hypothetic) concentrations in the seals associated with the herring PCB pattern was set at 0.01 μg/g. The logistic curve fitted through the data points had significant parameters (p < 0.001), and an EC50 of 7.6 μg/g was calculated. Approximately 93% of the data points for the harbor seals exceeded this EC50.

In Figure 3, the dose-response curves with Σ-PCB and metabolizable fraction are displayed for the pine marten and badger. The white-toothed shrew was used as a reference for the pine marten and the earthworm for the badgers. Both response
curves had significant parameters \( (p < 0.01) \), and the calculated EC50 was 1.6 and 0.6 \( \mu \text{g/g} \), respectively, for the marten and the badger.

**Blood samples**

The concentrations of \( \Sigma \)-PCB in blood samples from the Adélie penguin were the lowest of all species: 0.3 \( \mu \text{g/g} \) (Table 1). The relative concentrations of \( MF_{\text{pcb}} \), however, were the highest of all species. A PCA revealed that patterns of PCB congeners differed between periods (Fig. 4). At the moment of hatching, the pattern was relatively dominated by metabolizable congeners, whereas nonmetabolizable congeners dominated the pattern at the moment of crèche forming. The patterns of the various periods were significantly different from each other (redundancy analysis, Monte Carlo permutation tests, \( n_{\text{perm}} = 200, p < 0.005 \)). The \( MF_{\text{pcb}} \) also fluctuated significantly during the season (\( t \)-tests, \( p < 0.001 \)), with the \( MF_{\text{pcb}} \) concentrations being relatively high at the hatching and low at the crèche stage (Fig. 5), similar to the results of the pattern analysis.

**DISCUSSION**

The results of the predator and prey data are discussed first, followed by the hypothesis that \( MF_{\text{pcb}} \) is related in a dose-dependent manner to \( \Sigma \)-PCB. Finally, the applicability of blood samples as a basis for a nondestructive biomarker in PCB pattern analysis is reviewed.

**PCB congener pattern and metabolizable fraction**

Our results showed significant differences in congener patterns between predators and their prey in both terrestrial and aquatic food chains (Table 1). Prey species contained relatively more congeners with either \( o,m \)-vicinal H atoms and only one \( o \)-Cl atom or with just \( m,p \)-H atoms (open dots; Fig. 1). The substitution patterns of these congeners allow formation of an epoxide, which can be achieved by CYP isoenzymes [26]. This formation is the start of several major pathways in which PCBs can be metabolized [27]. The metabolism of PCBs by CYP isoenzymes via formation of an epoxide is not the obligate pathway [28], but it is assumed to be the most important [27]. Nevertheless, CYP isoenzymes can also metabolize PCBs by direct hydroxylation; therefore, depending on the specific set of isoenzymes involved, the treatment of the organism, and the structure of the PCB congeners, different CYP-related metabolic pathways may be involved in the metabolism of PCBs [28].

In accordance with harbor seals, badgers and pine martens contained relatively more nonmetabolizable PCBs (e.g., PCB 132, 153, 170, 180, and 194) compared with their respective prey species. In the terrestrial food chain, the relative concentrations of metabolizable congeners decreased in the following order: earthworm > white-toothed shrew > badger > pine marten. These results suggest some metabolic capacity in the white-toothed shrew and a relatively high capacity in the badger and pine marten. These differences could not be related, however, to the \( \Sigma \)-PCB concentrations in the different species. Even so, they may be related to differences between species in the patterns of available CYPs and their potential to metabolize certain PCBs [28]. Moreover, species-specific induction of these isoenzymes may play a role.

When CYP activity is induced, the fraction of all metabolizable PCB congeners generally decreases [11,17]. Hence, the reciprocal of the summed concentrations of all metabolizable congeners relative to \( \Sigma \)-PCB should reflect the metabolic CYP activity of the sampled animal [16]. This activity appears to be higher in predatory species (Fig. 1), which is indicated by the decreased metabolizable fraction of \( \Sigma \)-PCB in predators compared with prey (Table 1). In Figures 2 and 3, the \( \Sigma \)-PCB–dependent induction of the CYP metabolism of PCB congeners is modeled as a logistic curve. These curves show that intra-
species variation in the metabolizable fraction of PCB congeners among predators can be explained by exposure to PCBs. The dose-response curves show a direct link between a chemical signal and a biologic response that can be measured within a single analysis. In these curves, the PCB patterns of prey species are included as a reference to reflect the hypothetic situation of predators exposed to very low levels of PCBs not actually found in the current study (Figs. 2 and 3). This is done because predators exposed to low concentrations could not be included in the data set. Consequently, assessment of the upper asymptote of the curves is based on the $MF_{pcb}$ of the prey species. In this study, we used only one prey species as a model for the spectrum of prey on which a predator may forage. It is questionable, however, whether this correctly represents the PCB pattern of animals exposed to low concentrations. Furthermore, even predators exposed to low concentrations would exhibit some basal CYP activity. Hence, the metabolizable fraction of $\Sigma$-PCB in the prey species likely may be overestimating the $MF_{pcb}$ that would have occurred in predators exposed to low concentrations.

Combining these arguments, it was questionable whether the assessment of the upper asymptote and other parameters such as the EC50 was correct. To verify our method, we varied the input $MF_{pcb}$ of the different prey species. If the $MF_{pcb}$ in all herring samples was decreased by 5%, the EC50 for the harbor seal increased from 7.6 to 8.9 µg/g, which was not significant. When this $MF_{pcb}$ was decreased by 10%, however, the EC50 became 10.4, and this change was significant. In the
other species, no significant differences in EC50 were apparent at a 5 or a 10% decrease of the \( M_{F_{pcb}} \) among prey. Considering the absence of predators exposed to low concentrations, the use of a single prey species as a reference appears to be a useful, though not ideal, option. Therefore, it should be emphasized that ideally, predators exposed to low concentrations should be included in the analysis or, as an alternative, that a spectrum of prey species should be used.

Assessment of causative compounds

The dose-response curve can be used to assess which group of compounds is responsible for the metabolism of PCBs. When interpreting a dose-response curve (Fig. 3), three areas can be distinguished: (1) the area above the curve’s 95% confidence interval, (2) an area beneath this interval, and (3) the curve’s confidence interval itself. If an organism fits within the curve’s confidence interval, the CYP induction that occurs can be attributed solely to the PCB burden. Thus, in a further risk assessment, screening for other compounds that may have induced the CYP isoenzymes may be needed. Alternatively, the exposure to organochlorines that induce CYP activity, and the exposure to other xenobiotic compounds are inhibiting the CYP activity responsible for metabolism of PCBs.

CONCLUSIONS

A meta-analysis of the results of studies on congener-specific PCB analyses in several predator and prey species shows that metabolism of certain PCB congeners correlates with the concentration of total PCBs in the organism. This relation can be described by a logistic curve. Reduction of the metabolizable fraction of PCB congeners is suggested to reflect the induction of PCB-metabolizing enzymes, such as CYP1A1, which are generally regarded as being early warning signals for the Ah-receptor-related toxic effects of PCBs and related compounds. The PCB pattern analysis can also be applied to samples obtained nondestructively, as illustrated by our use of blood samples. This results in a nondestructive biomarker for the exposure to organochlorines that induce CYP activity, which is a great advantage over the biomarkers currently used, such as alkoxyresorufin-O-dealkylase measurements, which are generally destructive to the organisms studied or otherwise difficult to obtain. Furthermore, a PCB pattern analysis can indicate if PCBs are solely responsible for CYP induction or if other compounds also play a role. In addition, PCB pattern analysis can be applied in a retrospective manner using existing data sets or archived samples. Application of PCB pattern analysis on such material or data sets on PCBs may result in a review of the effects that organochlorines may have had on enzyme activity in organisms during previous periods. Hence, PCB pattern analysis appears to be a potentially valuable nondestructive biomarker, with various applications, linking CYP isoenzyme activity with PCB exposure in a single analysis.

Acknowledgement—This study was cofunded by a grant from the Platform alternatieven voor Dierproeven of the Netherlands Organization of Scientific Research, grant PAD97-26. Paul de Bie, Gerard Müskens, and Jos Bodt skillfully collected the samples from the terrestrial species used this study.

REFERENCES

2. National Research Counsel. 1989. Biologic Markers in Repro-
Nondestructive biomarker in PCB pattern analysis


