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THE CALUX (CHEMICAL-ACTIVATED LUCIFERASE EXPRESSION) ASSAY ADAPTED AND VALIDATED FOR MEASURING TCDD EQUIVALENTS IN BLOOD PLASMA

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Abstract—A method was developed to isolate lipophilic compounds efficiently from small aliquots of blood plasma and test these for total dioxin-like toxic potency using recombinant rat (H4IIE) and mouse (Hepa1c1c7) hepatoma cell lines, containing the firefly (*Photinus pyralis*) luciferase gene under trans-activational control of the aryl hydrocarbon receptor (AhR). For this experiment, blood plasma was used originating from eider ducks (*Somateria mollissima*) that had been dosed with 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) or with the technical PCB-mixture Clophen A50. For each sample the CALUX (chemical-activated luciferase expression) response of both the fat-containing organic extract and the fat-free, cleaned extract were compared with data from chemical analyses of these samples. The CALUX responses for the extracts were converted into so-called CALUX TEQs (TCDD equivalents), using a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard curve. The CALUX TEQs in both fatty and cleaned extracts correlated significantly with PCB-77 or PCB-153 levels (depending on the dosage group) determined in blood plasma using gas chromatography–mass spectrometry (GC-MS). For PCB-77 a toxic equivalency factor (TEF) of 1.5×10^{-3} was calculated based on these correlations. In addition, PCB-118 and PCB-156 levels in abdominal fat (assessed with GC with electron capture detection) and hepatic ethoxyresorufin *O*-deethylase activities correlated well with the CALUX TEQs in both fatty and cleaned blood plasma extracts, suggesting the TEQ levels in blood offer a good measure for internal dose. Plasma cholesterol and triglyceride levels were determined as a measure of lipid content, in 10-μl aliquots of blood plasma using enzymatic spectrophotometric determination. In conclusion, we have demonstrated that the CALUX assay is a rapid, sensitive assay for assessing the toxic potency of (mixtures of) AhR-active compounds in small aliquots of blood plasma. The limit of detection for the CALUX assay is currently less than 0.1 fmol (32 fg) TEQ, which corresponds with the amount of TEQs present in 0.1 to 1 ml of blood plasma in environmentally exposed species or man.

Keywords—PHAH Biomarker Lipids Blood plasma Reporter gene assay

INTRODUCTION

It is essential for proper risk assessment of micropollutants occurring in the environment to be able to measure the internal level of exposure of wildlife species. Quantification of the toxic potency of the whole mixture of compounds acting via a specific mechanism, instead of only a single or a few representatives, would strengthen the causal relationship between an observed adverse effect and the presence of a (group of) chemical(s).

Polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated biphenyls (PCBs) and polychlorinated terphenyls (PCTs), are ubiquitous pollutants that are especially associated with the aquatic food chain. The highest PHAH concentrations are found in top predators of the aquatic food chain, such as mussel- or fish-eating birds, and fish-eating mammals [1–4]. PHAHs elicit a number of species-specific, toxic responses in laboratory and wildlife species, including hepatoxicity, body weight loss, thymic atrophy, and impairment of other immune responses, dermal lesions, reproductive toxicity, alterations in vitamin A and thyroid hormone metabolism, teratogenicity, and carcinogenesis [5–12].

PHAHs are present as complex mixtures in environmental matrices such as sediments, wildlife, and humans. Industrial applications of some PHAHs, such as PCBs and PCTs, have been banned in industrialized countries since the early 1980s. They are, however, still entering the environment, e.g., by leakage from old, so-called closed systems, from recycling of old contaminated materials, leakage from dump sites, and long-range atmospheric transport and deposition [13]. The recent pattern of PCB contamination suggests their usage still continues in tropical countries [14]. Other PHAHs with qualities comparable to PCBs, such as polybrominated and polychlorinated diphenylethers (PBDEs and PCDEs), are still being produced and used [15]. Moreover, environmental input of PHAHs such as polychlorinated-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) is still continuing due to their formation during incomplete combustion in the presence of chlorine [16,17].

As PHAHs are lipophilic, persistent compounds they mainly accumulate in the lipid compartments of organisms. The distribution in the body is dependent on the structure and the physicochemical characteristics of the individual congeners. Excretion depends to a large extent on the metabolism to more polar compounds. The rate of metabolism of PHAHs depends upon halogen content and substitution pattern, the dose, and the animal species [18]. As a result, the absolute and relative abundance of individual congeners differs greatly between various biological samples [3,16].

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The mechanism of action for the toxic, planar PHAHs, which are approximate isostereomers of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been partially elucidated and involves a receptor-mediated process. After binding of the ligand to the cytosolar aryl hydrocarbon receptor (AhR), the ligand–receptor complex is activated and translocated to the nucleus, wherein it binds to dioxin-responsive elements (DREs), followed by stimulation of transcription of adjacent downstream genes [19]. Induction of cytochrome P450 1A1 and its associated increase in ethoxyresorufin *O*-deethylase (EROD) activity is often studied, because its AhR-mediated expression is altered in tandem with that of other enzymes and receptor proteins [8]. The use of the EROD activity for quantifying AhR-active compounds is, however, limited under several circumstances by the phenomenon of substrate inhibition [20,21].

For hazard and risk assessment of mixtures of PHAHs, the relative toxic potencies of individual PHAH congeners compared to TCDD have been transformed into toxic equivalency factors (TEFs). The concentrations of the individual congeners multiplied by their respective TEFs are added up to give the total TCDD toxic equivalency (TEQ) of the mixture [18,22]. However, the often small concentrations of individual congeners, the presence of unknown or not routinely measured AhR-active substances, the lack of TEF values for several PHAHs, and the possible additive and antagonistic interactions between PHAHs [10,23] are drawbacks to the TEQ approach. In addition, an extensive sample clean-up is needed for chemical analysis of the toxicologically relevant planar PHAHs, in order to separate them from the bulk of other congeners [9]. Therefore, a sensitive and quick prescreening assay is needed for monitoring the toxic potency of whole mixtures of PHAHs in relatively small samples, such as blood plasma.

The CALUX (chemical-activated luciferase expression) assay

A novel in vitro bioassay has been developed recently, based on AhR-mediated firefly (*Photinus pyralis*) luciferase gene expression in genetically modified cell lines [23,24]. The assay is called the CALUX assay. To produce the CALUX cells, a vector containing the luciferase gene under transcriptional control of DREs was stably transfected into mouse (Hepa1c1c7) and rat (H4IIE) hepatoma cell lines and several other cell lines [23–26]. Luciferase induction by TCDD appeared to be dose dependent, the current detection limit of the CALUX assay is less then 0.1 fmol TEQ, and the dose–response curve saturates at ligand concentrations greater than 100 to 1,000 nM, indicating the CALUX response is usable for a wide range of PHAH concentrations. For the PCDD-, PCDF-, and PCB-congeners tested so far, the relative potency to induce CALUX activity correlated well with reported TEF values [23,24,27,28].

Objectives of the present study

Most of the studies performed with the CALUX assay so far regarded responses induced by (combinations of) pure compounds, determination of TEF values for single compounds and detection limits, and comparison with the responses in the EROD assay. For environmental purposes it is necessary to develop and validate methods to extract PHAHs from environmental matrices and prepare these extracts for testing in the CALUX assay, as has been done for sediments and pore water [20]. The present study was performed to develop a fast and cost-effective method for extraction of PHAHs from small blood plasma samples and test these in the CALUX assay. The responses of both the raw and the cleaned (fat-free) extracts in the CALUX assay were compared, to be able to indicate consequences for use in environmental screening assays. The measured CALUX TEQs for blood plasma samples from eider ducks originating from a former experiment were compared with PCB levels in blood plasma to determine the extraction efficiency, and with PCB levels in abdominal fat and the hepatic EROD activity to get an indication whether the nondestructive determination of TEQs in blood offers a measure of the toxicologically relevant internal exposure. Plasma cholesterol and triglyceride concentrations were determined and used for normalization of PHAH levels on a lipid basis.

MATERIALS AND METHODS

Chemicals

Methanol, diethyl ether, and isooctane were of p.a. grade and purchased from Merck (Germany). Hexane was of high-performance liquid chromatography (HPLC) quality (Rathburn, Scotland). 2,3,7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Schmidt (the Netherlands) and the purity and concentration of the TCDD stock solution was reconfirmed by gas chromatography–mass spectrometry (GC-MS) analysis (Dutch State Institute for Quality Control of Agricultural Products, RIKILT-DLO). Ultraclean dimethyl sulfoxide (DMSO) was purchased from Janssen (Belgium). Sulfuric acid (z.a., Baker, Holland) was washed with hexane before use in a silica gel 60 (70–230 mesh, ASTM, Merck) column with dried sodium sulfate (p.a., Merck).

Eider ducks

In a former experiment [29], newly hatched eider ducklings were caught on the isle of Vlieland in the Dutch Waddenzee and kept in large open-air cages as described in Murk et al. [29]. After an acclimation period of 27 d the animals were dosed once intraperitoneally with the vehicle corn oil, 5 or 50 mg 3,3′,4,4′-tetrachlorobiphenyl (PCB-77)/kg body weight (bw), or with 50 or 200 mg Clophen A50/kg bw. Ten days later the animals were killed under ether anesthesia, and in addition to tissues, blood was collected and processed to obtain plasma. Blood plasma was stored at −20°C. EROD activities were measured in hepatic microsomes, and a number of mono-*ortho* and di-*ortho* PCB congener levels were determined in abdominal fat using GC with electron capture detection (ECD), as described in [30]. Lipids were extracted from abdominal fat using pentane and quantified gravimetrically. For the experiment described in this paper the blood of 25 of these animals was used for CALUX-TEQ and chemical analysis. Of the PCB levels in abdominal lipid already determined for these animals, only the levels of 2,3,4,4′,5-pentachlorobiphenyl (PCB-118) and 2,3,3′,4,4′,5-hexachloro biphenyl (PCB-156) were used for comparison with the bioassays, because these congeners contributed most to the total TEQs of the PCB mixture. For the PCB-77-dosed animals the PCB-77 levels in the abdominal fat were available.

Extraction and clean-up of blood plasma

A selection of 25 blood plasma samples was made based on the internal PCB concentrations measured in abdominal fat, in order to obtain a wide range in concentrations. Blood plasma aliquots of about 1.5 ml in hexane-washed glass centrifuge tubes were denaturated with one volume of methanol. After

vigorously mixing, two volumes of hexane were added (about 3 ml) and after mixing again, the tubes were centrifuged for 2 min at 1,500 *g* at room temperature. The hexane layer was transferred to another hexane-washed tube, and 1 drop of 6 M HCl per 0.5 ml of plasma was added to the remaining water phase. Subsequently, the samples were extracted twice again with hexane as described above. Finally 0.5 ml hexane was added to the water phase, collected again without mixing, and pooled with the rest of the hexane extract. The extraction efficiency of PHAHs from the blood plasma was not determined for each individual sample, as addition of ^{13}C-, or ^{14}C-PCB standards would influence the CALUX measurements. However, the average extraction efficiency was tested using ^{3}H-TCDD-spiked eider duck blood samples. The recovery was always 99 to 101%.

An aliqout of the hexane extract was taken for further cleanup and GC analysis, the rest was pipetted into a conical screw vial and evaporated at 30°C under a gentle stream of nitrogen gas. These extracts are further referred to as fatty extracts. Shortly before all hexane was evaporated, the desired amount of DMSO was added. After all hexane was evaporated a dilution series of the samples was made in DMSO. For the first dilution step the extracts had to be kept warm (±30°C) to prevent clotting of the lipids in the pipet. The fatty extracts were tested in 6-well plates using Hepa.Luc cells (see below).

The other part of the hexane fraction of the plasma extract was cleaned over a 20% H_2SO_4-deactivated silica column with 15 ml hexane–diethyl ether (97:3, v/v). This extract is further referred to as cleaned extract. The recovery of PHAHs from the silica–H_2SO_4 column could not be tested for each individual sample for the same reason as mentioned above. From former experiments however, using ^{13}C-PCB standards under the same standard conditions, we know that this recovery is more than 95%. The cleaned extract was evaporated to 0.5 ml; 1 ml isooctane was added and the extract was quantitatively transferred to a tapered vial and evaporated to approximately 50 μl for GC-MS determination (see below). After GC analysis, the remaining extract was evaporated, dissolved, and diluted in DMSO for CALUX measurement in 24-well plates using H4IIE.Luc cells (see below).

CALUX assay

H4IIE.pGudluc1.1 (H4IIE.Luc) or Hepa.pGudluc1.1 (Hepa.Luc) cells, prepared as previously described in [23,24] (also called respectively H4L1.1c4 and H1L1.1c7), were used for experiments performed in, respectively, 24-well or 6-well culture plates (Costar). All cells were grown in minimal essential medium (α-MEM, Gibco) with 10% heat-inactivated (h.i.) fetal calf serum (FCS, Gibco) and 500 μg of G418 (Geneticin, Gibco) per ml of medium, at a temperature of 37°C and 5% CO_2. The CALUX experiments with the fatty eider duck plasma extracts were performed in 6-well cell culture plates in 3 ml α-MEM per well. Due to technical improvements during the course of these studies, the cleaned extracts were tested in 24-well cell culture plates with 0.5 ml α-MEM per well, both without Geneticin. When the cell layer reached 90 to 100% confluency, 24 h after seeding, the cells were dosed with the test compounds in a maximum of 0.5% DMSO in growth medium by adding the DMSO to the culture medium (6-well plates) or replacing the medium with fresh culture medium containing the DMSO (24-well plates). Exposure was in triplicate, and on each 24-well plate three TCDD calibration standards, or a TCDD standard series per assay (6-well plates) were included. After 20 to 24 h of exposure the medium was removed and cells were washed twice with phosphate-buffered saline (PBS; Oxoid, UK). The cells were harvested in 250 μl (6-well plates) or 75 μl (24-well plates) cell lysis reagent (Promega), centrifuged for 3 min (6-well plates) or 90 s (24 wells plates) at 13,000 *g*, and the supernatant frozen at −80°C. For measurement of luciferase activity the supernatants were thawed on ice, 20-μl aliquots were pipetted into a 96-well microtiter plate, 100 μl luciferin assay mix (Promega) at room temperature was added, and the plate was mixed for approximately 90 s on a plate mixer (Amersham). The light production was measured in an Amerlite Luminometer (Amersham). The protein content of each supernatant was measured in a microtiter plate at 595 nm (Molecular Devices microplate reader), using Bio-Rad protein assay dye reagent and bovine serum albumin (BSA) as protein standard.

The GC-MS method

The cleaned eider duck blood plasma extracts were analyzed for PCB-77 (PCB-77-dosed animals) or PCB-153 levels (rest of the animals), using GC coupled to a Saturn II ion trap detector (ITD) (Varian, Walnut Creek, CA, USA). The GC column was a 30-m × 0.2-mm i.d. DB5-MS with a film thickness of 0.2 μm (J&W Scientific, Folsom, CA, USA). A retention gap of 2-m × 0.53-mm i.d. deactivated fused silica (Chrompack, Middelburg, the Netherlands) was used. The helium flow rate was 1.2 ml/min at 90°C (2 min) at 14°C/min to 240°C, next at 15°C/min to 270°C with a hold for 15 min at 270°C. For the identification and determination of PCB-77 and PCB-153 a cluster of masses was selected and a single mass was used for the quantification. Further GC and ITD conditions are described elsewhere.

Enzymatic triglyceride and cholesterol assays

Triglyceride concentrations were determined in 10 μl samples of blood plasma by quantitative enzymatic determination using commercially available (Sigma Diagnostics, procedure no. 337) triglyceride reagent (GPO-Trinder) and glycerol standard (250 mg/dl). This method is based on enzymatic hydrolysis of triglyceride, resulting in the production of a quinoneimine dye (measurable at 540 nm) directly proportional to glycerol released from triglycerides in the sample.

Cholesterol concentrations were determined in 10-μl samples of blood plasma by quantitative enzymatic determination using commercially available cholesterol reagent (Sigma Diagnostics, procedure no. 352) and standard (200 mg/dl). This enzymatic method also yields a quinoneimine dye (measurable at 500 nm) that is directly proportional to the total cholesterol concentration in the sample.

Calculations of CALUX TEQs and statistics

For calculation of CALUX TEQs a complete standard curve of 2,3,7,8-TCDD was used for each cell line. In each assay, three different TCDD calibration standards, bracketing the TCDD EC_{50}, were measured to correct for assay-to-assay variation. The standard curve was fitted (one-site ligand fit) using SlideWrite 5.1, and the CALUX TEQ value for an unknown sample was interpolated on this curve. For each sample a dilution series was made and measured in triplicate. The dilution of the sample that resulted in a CALUX response between the EC_2 and EC_{50} of the TCDD response, preferably close to the EC_5, was used for quantification of the sample. This is the most linear part of the dose–response curve, and

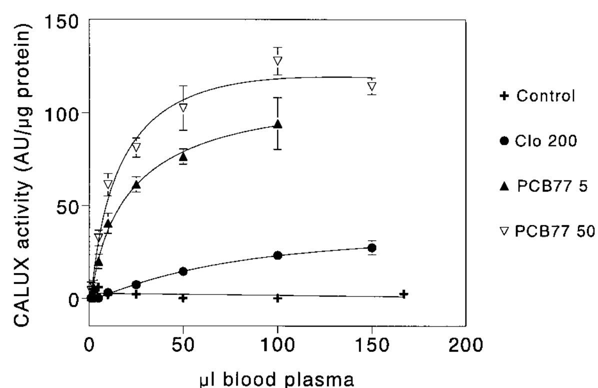

Fig. 1. CALUX (chemical-activated luciferase expression) induction in Hepa.Luc cells by fatty blood plasma extracts from a control, 200 mg Clophen A50/kg (CloA50 200), and a 5- or 50-mg 3,3′,4,4′-tetrachlorobiphenyl (PCB-77)/kg (PCB77 5 or PCB77 50)-dosed eider duck.

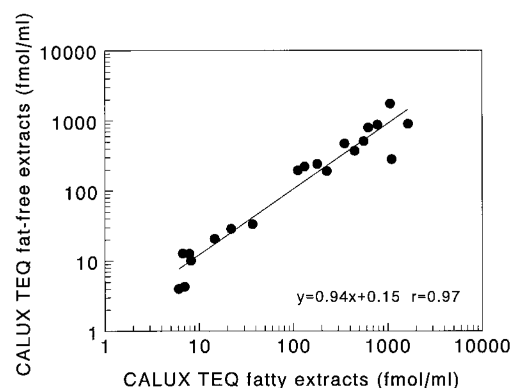

Fig. 2. Correlation between chemical-activated luciferase expression 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents determined for uncleaned, fatty blood plasma extracts from experimentally exposed eider ducks, measured in 6-well plates using Hepa.Luc cells, and the same extracts after clean-up, determined in 24-well plates, using H4IIE.Luc cells ($r = 0.97$). For details see Materials and Methods.

the quantifications based on this part of the curve are very reproducible.

Statistical analysis of dose–effect relationships was performed by unweighted least-squares linear regression analysis. Differences between group means were tested using one-way ANOVA. Both calculations were performed using Statistix version 4.0. The acceptance level was set at $p < 0.05$.

RESULTS

The dose–response curve for TCDD used as a standard in the CALUX assay saturated between 100 pM and 1 nM, the EC50 was 10 pM, and the standard deviation was generally ≤5%. The detection limit in H4IIE.Luc cells was less than 1 pM TCDD, which equals an absolute amount of less than 0.5 fmol/well in 24-well plates. A full dose–response curve was presented before [20].

Dilution series of extracts from eider duck's blood plasma induced CALUX activities in a dose-related manner, according to a one-site ligand dose–response curve. Figure 1 presents dose–response curves for the fatty extracts from plasma of three eider ducks exposed to either 5 or 50 mg PCB-77/kg bw or 200 mg Clophen A50/kg bw, or from a corn oil-treated control animal. The CALUX activities induced by blood plasma extracts from animals dosed with 50 mg Clophen A50 /kg bw (not in the figure) were almost as low as the controls. The standard deviations (SDs) in the measured CALUX activities were 5 to 10%. The CALUX TEQs determined for the cleaned extracts in 24-well plates correlated very well with the CALUX TEQs, measured in 6-well plates, for the fatty blood plasma extracts (Fig. 2; $r = 0.97$).

The PCB-77 (for PCB-77 dosed eider ducks) or PCB-153 (for Clophen A50 dosed eider ducks) levels chemically measured in blood plasma correlated significantly with, respectively, PCB-77 levels ($r = 0.98$, $p < 0.001$) or PCB-118 or PCB-156 (respectively, $r = 0.96$, $p < 0.001$ and $r = 0.99$, $p < 0.001$) chemically measured in abdominal fat (data not shown). Correlations between CALUX TEQs determined in fatty or cleaned extracts with PCB levels determined in abdominal fat or blood plasma are presented in Table 1. The correlations between PCB-77 (PCB-77-dosed eider ducks), or PCB-118 and -156 levels (for Clophen-dosed eider ducks) in abdominal fat and the CALUX TEQs determined in the cleaned extracts are presented in Figure 3. The three control animals are left out of this figure because the PCB levels were at, or below, the detection limit with the dilution factors used in this experiment. The relationships between the PCB-77 or PCB-153 levels in blood plasma and the CALUX TEQ are comparable (data not shown). From the correlation between the TCDD equivalents measured with the CALUX assay and the chemically determined PCB-77 levels in blood plasma the TEF for PCB-77 in the CALUX assay was calculated ([CALUX TEQ]/[PCB-77]). Based on the CALUX data for fatty extracts, this TEF is 1.6×10^{-3}, based on the cleaned extracts the TEF is 1.5×10^{-3}.

The CALUX TEQs correlated significantly with the hepatic EROD activity ($r = 0.88$, $p < 0.05$ for the fatty blood plasma extracts, data not shown). For each exposure group, the average CALUX TEQs, hepatic EROD activity, and cholesterol and triglyceride levels in blood plasma are presented in Table 2.

Table 1. Correlations between chemical-activated luciferase expression 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (CALUX TEQs) and PCB levels in abdominal fat or blood plasma after logarithmic transformation

	Abdominal fat			Blood	
	PCB-118	PCB-156	PCB-77	PBC-153	PCB-77
CALUX TEQ (fatty)	$r = 0.86$ $p = 0.01$	$r = 0.08$ $p = 0.02$	$r = 0.94$ $p < 0.001$	$r = 0.81$ $p < 0.02$	$r = 0.95$ $p < 0.001$
CALUX TEQ (cleaned)	$r = 0.82$ $p < 0.01$	$r = 0.82$ $p < 0.01$	$r = 0.97$ $p < 0.001$	$r = 0.84$ $p < 0.01$	$r = 0.96$ $p < 0.001$

The three control animals are left out of these correlations because PCB levels were at, or below, detection limit.

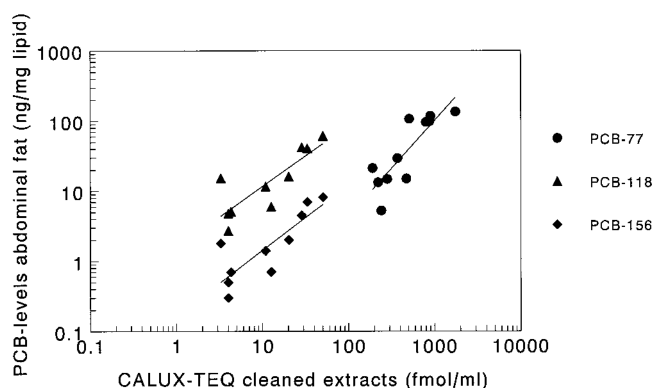

Fig. 3. Correlation between polychlorinated biphenyl (PCB)-118 or PCB-156 levels (for Clophen-dosed eiders) or PCB-77 levels (for PCB-77-dosed eiders) determined in abdominal fat using gas chromatography electron capture detection and the chemical-activated luciferase expression 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (CALUX TEQ) of cleaned blood plasma extracts determined in 24-well plates using H4IIE.Luc cells. For correlations see Table 1.

Average plasma cholesterol and triglyceride levels were not statistically significantly different, although the data suggest a slight increase in triglyceride levels and a decrease in cholesterol levels in the high-dose Clophen A50 animals (Table 2). All CALUX TEQs mentioned above were expressed on a volume basis, as fmol TEQ/ml blood plasma. When expressed on a triglyceride or cholesterol basis, the correlations with chemical data or EROD activity were comparable or slightly less (data not shown).

DISCUSSION

The CALUX TEQ in blood plasma as a measure of PHAH exposure

The combination of the extraction and clean-up method presented and the CALUX assay proved to be a sensitive, fast, and relatively easy method to determine the total toxic potency of mixtures of PHAHs in blood plasma, expressed in TCDD equivalents (TEQs). The CALUX TEQ in blood plasma represents an integrated measure of the internal dose of the AhR-active PCBs in eider ducks, experimentally exposed to either PCB-77 or Clophen A50. The independently performed CALUX assays in 6-well and 24-well plates resulted in almost identical CALUX TEQs, confirming the high reproducibility that was already observed in former experiments with pure compounds [20,24,28]. Based on the chemically determined PCB-77 levels and CALUX TEQs in blood plasma of PCB-77-dosed eider ducks, a relative potency of 1.5×10^{-3} was estimated. This value is in the range of the TEFs determined before in H4IIE.Luc cells: 0.7×10^{-3} [28] and 1.4×10^{-3} (Murk, unpublished data), and the TEF value of 0.01 proposed by Safe [10] and 0.5×10^{-3} as proposed by the WHO [31].

The results of this study demonstrate that blood plasma extracts can be measured equally well in the CALUX assay with and without application of a clean-up procedure. Skipping the latter step makes the analysis faster and reduces the probability of unforseen loss of yet unknown compounds of interest on the silica column. However, handling of the fatty extracts, such as in making a dilution range or concentrating the extract, may be more complicated because of the possible precipitation of lipids, unless the extract is carefully kept at about 30°C. In addition, adding a lot of lipid to the growth medium would gradually reduce the availability of PHAHs for the cells. Performing a simple additional silica–H_2SO_4 clean-up step circumvents these problems and allows one to concentrate the extract. It is also possible to expose CALUX cells directly to untreated blood plasma or serum (data not shown). Although it is tempting to use blood plasma samples as crude as possible for fast prescreening, recent information suggests that endogenous ligands, or compounds originating from food, may interfere with the response induced by PHAHs (Murk, unpublished results). As these factors may differ depending on food composition, species, sex, age, or health status, the results may be misleading.

Measure of lipid content for normalization

The observed good correlation of the CALUX TEQ in blood plasma with the GC-TEQ in abdominal fat suggests that PHAHs are relatively evenly distributed over the total lipid phase in a body. PCBs have been reported to be present in virtually identical levels in liver, heart, fat tissue, kidney, and muscle if expressed on a lipid basis. Higher concentrations were present in the liver [3] only of relatively biodegradable PCBs. Liver deposition appears to be relatively more important for non-*ortho* congeners, but the ratio between liver and adipose tissue concentrations are species dependent [18]. As blood plays an important role in the transport of PCBs between tissues, it is to be expected that there is a dynamic balance between PCB levels in blood, liver, and other organs. Therefore levels in blood can be used as an overall measure for the internal PHAH dose if normalized on a lipid basis [32,33].

Table 2. Chemical-activated luciferase expression (CALUX)-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TEQs) in plasma or abdominal fat of control and polychlorinated biphenyl (PCB)-exposed eider ducks; data are expressed as means ± SD

		PCB-77		Clophen	
Parameter	Control[a]	5 mg/kg[b]	50 mg/kg[c]	50 mg/kg[b]	200 mg/kg[c]
CALUX TEQ (fatty) (fmol/ml)	8.2 ± 6.4	398 ± 401	854 ± 443[d]	9.2 ± 4.6	37.8 ± 43.2
CALUX TEQ (fat-free) (fmol/ml)	11.5 ± 8.6	282 ± 112	870 ± 482[d]	12.4 ± 6.7	53.3 ± 81.1
Hepatic activity (nmol/mg protein min)	6.7 ± 2.7	59.4 ± 6.4	193.8 ± 126.9[d]	8.2 ± 5.9	14.4 ± 13.9
Cholesterol (mg/ml)	26.7 ± 1.7	26.7 ± 2.2	26.3 ± 2.9	27.9 ± 2.3	23.9 ± 4.1
Total triglycerides (mg/ml)	6.6 ± 4.5	6.5 ± 4.4	6.4 ± 1.8	6.9 ± 1.4	7.7 ± 1.4
True triglycerides (mg/ml)	4.8 ± 1.5	4.8 ± 0.6	5.0 ± 1.5	5.1 ± 1.7	5.8 ± 1.2

[a] $n = 3$.
[b] $n = 5$.
[c] $n = 6$.
[d] Significantly different from control with $p < 0.05$.

The brain is the only tissue with deviating PCB levels when expressed on a lipid basis, probably because the basic constituents of the cerebral lipids are phospholipids, in addition to the heme–encephalic barrier that blocks the passage of pollutants to a certain degree [32]. Phospholipids are characterized by a greater polarity than triglycerides and cholesterol and therefore do not play a great role in the retention of apolar PHAHs. As levels of PHAHs seemed to be linked essentially to triglycerides, standardization should be preferably on the basis of triglyceride weight instead of extractable weight, as levels of phospholipids differ among and within tissues. Other studies normalize on the basis of triglycerides and cholesterol (so called core fat) [33]. Effects on total plasma lipid levels of fasting and feeding are mainly caused by changes in triglyceride levels [34]. However, most laboratories quantify extractable lipids or total lipids gravimetrically. The resulting lipid determinations are very nonspecific and differ greatly between laboratories, depending on the extraction conditions and solvents used [35]. One of the problems is that the extracts contain other extractable material as well as lipids. Thus, measurements are extractable weight rather than only extractable lipid.

The advantage of the kits we used in this study for cholesterol and triglyceride measurement is that lipids can be specifically quantified in very small aliquots of plasma. In addition, quantification of categories of lipids could give additional information on the physiological condition of organisms. In this study, correlations between CALUX TEQs in blood and PCB levels in abdominal fat did not improve when expressed on a triglyceride or cholesterol basis instead of per ml of plasma. In addition, no differences were observed in triglyceride and cholesterol levels in blood plasma between the exposed eider ducks and the control group. The eider ducks used for this study were of the same age, lived under the same circumstances, and were fed the same food. Therefore, their plasma lipid levels were not expected to differ greatly, as was confirmed by the cholesterol and triglyceride measurements. However, when PHAH levels of naturally exposed individuals are to be determined, normalization on a lipid basis is necessary because for these animals large differences ecological and physiological conditions are to be expected, and lipid composition and lipid levels are influenced by nutritional status, condition, season, maturation, reproductive cycle, sex, species, organ, etc.

Perspectives for CALUX analysis of blood from environmentally exposed species

The experimentally dosed animals used for this study contained relatively high levels of PCBs. The main use for the CALUX assay, however, lies in the application for naturally exposed animals including humans. In the 24-well plates used in this study the detection limit is less than 0.5 fmol TEQ/well. In a recent integrated ecotoxicological study to establish otter-based quality standards for PHAHs [36], the TEQs in blood from naturally exposed otters (*Lutra lutra*) were determined using the CALUX assay and ranged from 4 to 200 fmol/ml (1–60 pg TEQ/ml) blood. The CALUX TEQs in the livers of these otters and the TEQ based on mono- and non-*ortho* PCBs correlated very well, and also absolute TEQ levels were comparable [21]. One milliliter of relatively clean otter blood (or 2 ml to prepare 1 ml of blood plasma) is needed to make a small dilution series, to be sure that one of the CALUX responses falls within the linear part of the TCDD dose–effect curve. For semiquantitative information such as relatively clean or highly exposed, only one sample needs to be measured (in triplicate); therefore about 0.3 to 0.5 ml blood plasma is needed. In recent studies, the CALUX assay has been further optimized and is currently performed in 96-well plates without the need of pipetting cell lysate and with automated injection of substrate in an automated luminometer (Labsystems) [21]. Not only does this further reduce the amount of time and material needed for the CALUX assay, it also decreases the limit of detection five times compared to the method presented here. The limit of detection of the improved CALUX assay is less than 0.1 fmol TEQ/well, resulting in a reduction of the amount of blood plasma needed for quantification of the CALUX TEQ to 0.1 to 0.5 ml (1 ml in relatively unexposed species).

Concluding remarks

The results demonstrate that the CALUX assay is a rapid and sensitive method to determine the AhR-related toxic potency of compounds present in blood plasma. The usefulness of the CALUX assay is especially evident when, due to small sample size or small concentrations of individual congeners, samples would have to be pooled, thus losing information, or animals have to be killed to get enough material. The CALUX assay can also be used for rapid screening of large quantities of samples. Samples inducing a great CALUX response could subsequently be chemically analyzed to identify the responsible compound(s).

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