Comparison of Chemical-Activated Luciferase Gene Expression Bioassay and Gas Chromatography for PCB Determination in Human Serum and Follicular Fluid

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We assessed exposure to dioxin-like compounds using chemical and bioassay analysis in different matrices in a female population. A total of 106 serum and 9 follicular fluid samples were collected from infertile women attending Centers for Reproductive Medicine in Belgium from 1996 to 1998. Major polychlorinated biphenyl (PCB) congeners were quantified by chemical analysis using gas chromatography with electron-capture detection, and the chemical-activated luciferase gene expression (CALUX) bioassay was used to determine the total dioxin-like toxic equivalence (TEQ) of mixtures of polyhalogenated aromatic hydrocarbons present in body fluids, such as serum and follicular fluid. To the best of our knowledge, this is the first investigation to determine TEQ values by the CALUX bioassay in follicular fluid. The TEQ levels in both matrices are well correlated ($r = 0.83$, $p = 0.02$). As the chemical and bioassay analysis executed in this study do not cover the same span of polyhalogenated aromatic hydrocarbons, we did not expect totally correlated results. Moreover, the sample workup and quantification of the analytes differed completely. Nonetheless, the TEQ values in human extracts correlated well with the sum of four major PCB congeners chemically determined in both serum and follicular fluid. These results indicate that the CALUX bioassay may serve as a simple, relatively inexpensive prescreening tool for exposure assessment in epidemiologic surveys. *Key words*: CALUX bioassay, chemical-activated luciferase gene expression bioassay, dioxin-like compounds, follicular fluid, polychlorinated biphenyls (PCBs), serum, toxic equivalences. *Environ Health Perspect* 108:553–557 (2000). [Online 2 May 2000]
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Polyhalogenated aromatic hydrocarbons (PHAHs) represent a class of widespread environmental contaminants including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs). Due to their low biodegradation and lipid solubility, these ubiquitous pollutants bioaccumulate throughout the food chain and may pose a health risk to both animals and humans (*1*). In fact, several epidemiologic studies indicate subtle changes in neurobehavioral and endocrine parameters in infants in relation to relatively high background levels of PHAHs, which can occur in industrialized countries in the Western World (*2,3*).

Since 1970, body burden measurements for chlorinated contaminants in humans have been determined in blood, follicular fluid, human milk, and adipose tissue (*4,5*). Current techniques for detection and quantification of PHAHs involve costly and time-consuming traditional chemical analysis by gas chromatography with mass spectrometry in selected ion monitoring or electron-capture detection mode. Gas chromatography analysis provides information about the presence and concentration of known isomers and congeners, but no information about the biologic effects of these complex mixtures (*6*).

Extensive experimental studies have revealed that most toxic actions induced by dioxin-like compounds are mediated via the aryl hydrocarbon receptor (AhR) signal transduction pathway (*7,8*). Quantification of the toxic potency of the whole mixture of compounds acting via the AhR pathway would strengthen the causal relationship between observed adverse effect and the presence of PHAHs as a group (*9*). For this purpose, the dioxin-like toxic equivalency factor (TEF) concept was introduced by Safe (*10*), allowing conversion of the PHAH chemical data set into the AhR-related toxic potency of the mixture of PHAHs. PCB congeners that can assume a coplanar configuration (non-*ortho*, and to a lesser extent some mono-*ortho* substituted congeners) also have a high affinity to the AhR and with binding initiate biologic actions and toxicity similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; the most toxic dioxin). Concentrations of individual PHAHs are multiplied by their respective TEF values and added together to give the dioxin-like total toxic equivalency (TEQ) value (*7,11*). It has recently been shown that the contribution of dioxin-like PCBs to the total TEQ value in human matrices is almost equal to the PCDDs/PCDFs fraction (*12,13*).

Recently, bioassays have been developed that can measure the total TEQ value of complex mixtures directly, without the need for extensive cleanup and chemical analysis procedures. One of the novel *in vitro* reporter gene assays, the chemical-activated luciferase gene expression (CALUX) bioassay, is based on AhR-mediated firefly (*Photinus pyralis*) luciferase expression in genetically modified cell lines (*6,14*). A vector containing the luciferase gene under transcriptional control of dioxin-responsive elements was stably transfected into a number of cell lines, including the rat H4IIE hepatoma cell line. The CALUX bioassay has recently been adapted and validated in blood plasma (*9*) and bovine milk (*15*).

In this study, we assessed exposure to PHAHs using a combination of chemical and bioassay analysis in a selected female subpopulation. Major PCB congeners in serum and follicular fluid were quantified by chemical analysis using gas chromatography with electron-capture detection (GC-ECD) and TEQ values in blood serum were determined using the CALUX bioassay. Four PCB congeners, IUPAC (International Union of Pure and Applied Chemistry) numbers 118, 138, 153, and 180, were selected because many European government and regulatory bodies use them as marker compounds to monitor occurrence and distribution. In human matrices, the three latter congeners make up 55% of the total PCBs (*16*). The congeners under chemical analysis are non–dioxin-like (only PCB-118 has a TEF-value of 0.0001) (*17*). The physicochemical characteristics and environmental behavior of individual PCB congeners are remarkably similar and have been found as complex mixtures in almost all compartments of the biosphere, including animal and human tissues and body fluids (*3*). Hence, we are exposed to a broad spectrum of PCB congeners with (non)–dioxin-like capacities.

To the best of our knowledge, this is the first investigation that aimed to determine TEQ values in follicular fluid. We decided to select infertile women as a study population. Some of these patients were candidates for donation of follicular fluid, as they were undergoing *in vitro* fertilization (IVF)

treatment. In addition, both analytical tools were applied in a case–control study to assess the impact of dioxins and related compounds on the occurrence of endometriosis. The main finding of this survey constituted the association of exposure to dioxin toxic equivalences with the risk for endometriosis [crude odds ratio (OR) = 4.3]. After adjusting for body mass index and alcohol consumption, the risk increased slightly to OR = 4.6. There was no confounding by age, ovulatory dysfunctions, caffeine intake, or smoking. On the other hand, there was no evidence for impact of exposure to four major PCB congeners on the occurrence of endometriosis (ORs ranging from 1.007 to 1.026, none of them significant). Therefore, the reported association between exposure to dioxins and related compounds and endometriosis cannot be confounded by the exposure to major PCBs (18).

Materials and Methods

Subjects and sampling. Subjects were all infertile females visiting a fertility unit. A couple is defined as "infertile" when pregnancy has not been achieved after 1 year of unprotected sexual intercourse. Sampling of serum and follicular fluid was performed at the Centers for Reproductive Medicine of the University Hospitals of Antwerp (UZA), Ghent (UZG), and Leuven (UZL). This study has been accepted by all ethical committees (protocol nos. 96/44/107, 97/100, and ML 536 for UZA, UZG, and UZL, respectively). All patients acknowledged their participation by signing an informed consent. The mean age of the women was 31.9 ± 4.0 years (range 24–42).

We collected 25 ml blood per patient in a vacuum system tube and centrifuged it (15 min, 2,000*g*) within 24 hr after collection. All serum samples were stored at -20°C until analyzed. Nine patients receiving IVF treatment were also candidates for donation of follicular aspirates obtained for investigation. Follicles were aspirated under ultrasound-guided transvaginal puncture after pituitary desensitization with Buserilin nasal spray (800 μg daily; Suprefact, Hoechtst, Belgium) followed by human menopausal gonadotropin (hMG), only 2 ampules of 75 U daily until at least two follicles reached a diameter of 20 mm. The puncture was performed 36 hr after an ovulatory trigger with 10,000 IU human chorionic gonadotropin (hCG) intramuscularly. After removal of the oocytes, all follicle fluids (free of blood contamination and granulosa cells) were pooled per patient and stored at -20°C until further analysis.

Chemicals and reagents. All solvents used for chemical analysis [pesticide grade (p.a.)] were purchased from E. Merck (Darmstadt, Germany). Four marker PCBs (IUPAC nos.

118, 138, 153, and 180) were a standard mixture from J.T. Baker (Deventer, The Netherlands) at a concentration of 10 ng/μL in isooctane. A standard mixture solution containing all compounds under investigation was prepared in *n*-hexane. The $^{13}C_{12}$-labeled CB-110 and CB-149 were obtained from Cambridge Isotope Laboratories (Woburn, MA).

For CALUX analysis, isopropanol, diethyl ether, and isooctane were of p.a. grade (Merck). Hexane was of HPLC quality. Ultraclean DMSO was purchased from Janssen (Beerse, Belgium). Sulfuric acid (AR, J.T. Baker) was washed with hexane before use in a silica gel 60 (70–230 mesh, ASTM; Merck) column with anhydrous dried sodium sulfate (p.a., Merck).

Chemical analysis by GC-ECD. Complete details of the solid-phase disk extraction are described and evaluated elsewhere (19). The method was shown to be consistent at three fortification levels. Reproducibility, in terms of relative standard deviation, was < 13% for all tested PCB congeners at the lowest fortification level (625 pg of each congener/g serum). Mean recovery for $^{13}C_{12}$ CB-110 (surrogate standard) was 85.0 ± 6.6%. A brief description of the method is given below. The sample preparation involved disruption of protein binding in an ultrasonic bath using formic acid (1:1, v/v), extraction, and concentration of analytes using Empore C18 SPE disk cartridges (3M, St. Paul, MN). A mixture of ethyl acetate and hexane was used for elution. Further cleanup of lipid interferences was accomplished using a sulfuric acid wash of the eluate. After concentration under a gentle nitrogen stream, we injected 2 μL per extract in splitless mode onto a GC-μECD system (HP 6890; Palo Alto, CA) by an autosampler (HP 7673). A fused silica DB-XLB capillary column (J&W Scientific, Folsom, CA) of 60 m × 0.25 mm i.d. × 0.25 μm film thickness was used with the following temperature program: initial 65°C held for 2 min, then to 220°C at a rate of 50°C/min, 1.5°C/min to 255°C, and then 20°C/min to 290°C, and held for 5 min. The carrier gas was hydrogen at a constant pressure of 135 kPa. The μECD temperature was kept at 320°C, using argon–methane (95 + 5) makeup gas at a flow rate of 20 mL/min.

CALUX bioassay. The extraction and cleanup procedure was performed as described in detail by Murk et al. (9). The method involves esssentially *n*-hexane extraction of blood serum or follicular fluid aliquots (1–1.5 mL) and removal of acid-labile matrix components by passage through a silica column containing 33% (w/w) concentrated H_2SO_4. This extract was quantitatively transferred to a tapered vial,

evaporated, and diluted in DMSO for CALUX measurement in 96-well plates using H4IIE.Luc cells.

Rat H4IIE hepatoma (H4L1.1c4) cells stably transfected with an AhR-controlled luciferase reporter gene construct (pGudluc1.1) were grown to confluence in 96-well view plates and exposed in triplicate to the PHAH samples and TCDD standards for 24 hr, using DMSO (0.5% v/v) as a vehicle. After removal of the medium, cells were washed twice with phosphate-buffered saline (Oxoid, Hampshire, UK). The cells were harvested in 75 μL cell lysis reagent and centrifuged, and the supernatant was 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, and 100 μL luciferin assay mix at room temperature was added. After thorough mixing, we measured the light production in a Luminoskan RS Luminometer (Labsystems, Helsinki, Finland).

Lipid determination. Lipids of serum and follicular fluid were determined enzymatically. Concentrations of triglycerides, cholesterol (free and cholesterol esters), and phospholipids were determined in duplicate by quantitative enzymatic determination using commercially available reagents from Elitech Diagnostics (Sées, France). We calculated the total serum lipid concentration by adding the amounts of triglycerides, cholesterol, and phospholipids. The total mean serum lipid concentration by enzymatic determination was 507 ± 122 mg/dL (*n* = 106, range 214–1,059). For the follicular fluids, the cholesterol and triglycerides could not be quantified enzymatically. Hence, the phospholipid contents were used for further calculations. The mean lipid concentration for follicular fluids was 49.2 ± 15.8 mg/dL (*n* = 9, range 23–72).

Calculations of CALUX TEQs and statistics. We calculated CALUX-based TEQs by comparing the luciferase activity induced by the sample against a dose–response curve generated from TCDD concentration standards analyzed simultaneously. The standard curve was fitted (one-site ligand fit) using SlideWrite 6.00 (Advanced Graphics Software Inc., Encinitas, CA), and the CALUX TEQ value of an unknown sample was interpolated on this curve. A full dose–response curve was presented previously (20). Mean values were calculated from the individual TEQ/g fat values thus obtained from each of three replicates.

All statistical calculations were performed using Statistica (version 5; StatSoft, Tulsa, OK). Means, SDs, and medians are reported in terms of original distributions. All the variables were tested for normality by Shapiro-Wilk's *W*-test. We calculated associations

between independent variables using Pearson correlation coefficients. A finding was considered statistically significant if its p-value was < 0.05.

Results

Residue levels in serum and follicular fluid.
PHAH body burdens were determined in human serum and follicular fluid obtained from infertile women attending one of the collaborating Centers for Reproductive Medicine. A summary of PCB congener levels, achieved by chemical analysis, is listed in Table 1. The arithmetic means ± SDs and medians, together with the number of samples containing quantifiable amounts, are presented. Missing data derive from peak interferences in the gas chromatogram. The values indicate that the concentrations for individual PCB congeners are higher in follicular fluid, partly due to the lower fat content of this fluid. If the results are expressed on a whole-weight basis (data not shown), the numbers fall in the same range for both matrices.

To evaluate how accurately the serum concentrations would predict the follicular fluid levels, correlations between the values were computed to determine their strength of association for each congener. Except for PCB-118, the numbers in Table 1 reveal strong and statistically significant correlations for the individual congeners and the sum of PCBs in both matrices.

To compare the PCB accumulation pattern in follicular fluid and serum, the total sum of all four congeners has been taken as 100%, and this distribution is presented in Figure 1. Follicular fluid and serum show opposite patterns of accumulation for PCB-153 and PCB-180. PCB-153 is the major congener in serum, in contrast to the follicular fluid, where PCB-180 is considerably more concentrated.

TEQ values measured through the CALUX bioassay are summarized in Table 2. The results of 14 serum samples and 1 follicular fluid sample were below the limit of

detection (32 fg TEQ/well). The mean TEQ level in follicular fluid is considerably higher than the mean serum TEQ value (257.9 ± 225.0 and 46.8 ± 43.8 pg TEQ/g lipid, respectively, in follicular fluid and serum), partly due to the lower fat content of the follicular fluid. However, the correlation between TEQ levels in serum and follicular fluid is statistically significant ($n = 7$, $r = 0.83$, $p = 0.02$). The high coefficients of variance are partly due to the small numbers (follicular fluid and plasma) and partly due to a wide variation of contamination in these selected subpopulations.

Comparison between CALUX TEQ values and PCB levels. The Pearson correlation coefficients between the CALUX TEQ values and the nonplanar PCB congeners within the serum samples are all highly significant and vary from 0.34 to 0.43 (Table 3). In addition, the correlation coefficients in follicular fluid are up to 0.74 for the PCB congeners. The correlation is statistically significant for PCB-138. The distributions of the sum of PCB congeners and TEQ levels in serum are shown in Figure 2A and 2B, respectively. Both skew distributions show a median level lower than the mean concentration.
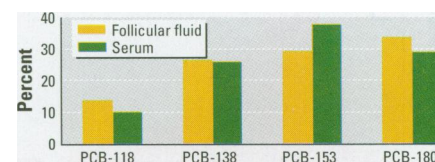
Comparison of TEQ levels with other CALUX data. In 1989, a collaborative study was started in The Netherlands (Dutch Mother's Milk Cohort Study); this study concerning the potential effects of PCBs and dioxins on the growth and development of newborns (21–23) was supported by the Dutch Toxicology Research Promotion Programme and the Health Research Stimulation Programme. The CALUX data of the present investigation can be compared with plasma samples, derived from the Dutch Mother's Milk Cohort Study, determined under the same analytical conditions. These 12 plasma samples were taken between 1990 and 1992 from nursing mothers; therefore, the patients' ages are presumably comparable. The values in Table 2 indicate that the Dutch women sampled approximately 6 years before this investigation had a considerably higher

level of exposure (mean 103.7 ± 51.5 pg TEQ/g lipid) than the Belgian female patients recruited for the present survey (mean 46.8 ± 43.8 pg TEQ/g lipid). The lipid contents of the samples of the Dutch survey were determined gravimetrically, whereas the recent CALUX results are all based on enzymatic lipid determinations.

Discussion

Currently, most toxicologic risk assessments are based on chemical residue analysis correlating them with determinations of toxic effects in wildlife species and humans. Even if the toxicities of all compounds were known, it would not be possible, on the basis of a chemical analysis alone, to say anything definite about how the pollutants in the sample affect living organisms (24). Because dioxin-like compounds normally exist in environmental and biologic samples as complex mixtures of congeners, the concept of TEQs has been introduced to simplify risk assessment and regulatory control. In applying this concept, relative toxicities of dioxin-like compounds in relation to TCDD (i.e., TEFs) are determined based on in vitro and in vivo studies (11). Previously, TEQs were determined using gas chromatography with high-resolution mass spectrometry (GC-HRMS) for the chemical analysis. Although this approach is an improvement over simple chemical analysis, 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/or antagonistic interactions between PHAHs (1,11) are drawbacks to this GC-HRMS analysis (9). These pitfalls are overcome by the immediate measurement of the biologic response instead of determining the culprit compounds using chemical analysis. This alternative technique using the CALUX bioassay determines the TEQ value as a sum of the synergistic, additive, or antagonistic interactions of all AhR-active compounds.

In this survey, exposure assessment to dioxin-like compounds was performed using gas chromatography to quantify PCB congener levels and the CALUX bioassay, which determines the total dioxin-like toxic equivalence of mixtures of PHAHs as present in

Table 1. PCB congener levels determined by GC-ECD in Belgian female body fluids, 1996–1998.

	PCB-118	PCB-138	PCB-153	PCB-180	Sum PCBs[a]
Serum ($n = 106$)					
No. positive[b]	101	98	102	101	97
Mean ± SD	27.7 ± 16.2	69.9 ± 30.7	94.5 ± 45.2	72.0 ± 35.7	263.5 ± 115.6
Median	25	64.7	85.3	62.7	245.7
Follicular fluid ($n = 9$)					
No. positive	9	9	9	9	9
Mean ± SD	149.4 ± 77.1	329.7 ± 223.5	352.4 ± 199.6	406.5 ± 235.9	1,238.0 ± 694.0
Median	134.2	242.8	346.9	428.9	1,288.6
Correlation, serum–follicular fluid					
No. samples	9	9	9	9	9
Correlation	0.40	0.66	0.71	0.73	0.71
(p-Value)	0.28	0.05	0.03	0.03	0.03

All values are expressed in ng/g lipid.
[a]Sum PCBs is the sum of the four congeners. [b]Lower numbers indicate missing data due to interferences.

Figure 1. Distribution pattern of PCB congeners in follicular fluid and serum ($n = 9$) according to their fractions. The concentration of the sum of PCBs was set at 100%.

body fluids such as serum and follicular fluid. Unfortunately, the bioassay results to identify the culprit dioxin-like compounds could not be confirmed by chemical analysis. Due to the low levels in biologic matrices, the chemical analysis of dioxins and related compounds requires a multistage cleanup and sensitive and selective detectors (high resolution or negative ion chemical ionization mass spectrometry). In addition, the large amounts of human sample needed (50 mL serum) for quantification were unacceptable in the current study for ethical reasons. In contrast, the sample amount needed for the CALUX assay was limited to 1.5 mL/patient.

Because the chemical and bioassay analyses presented here do not cover the same range of PHAHs, we do not expect totally correlated results. Moreover, the sample workup (extraction and further purification) and quantification of the analytes were completely different. Nonetheless, the TEQ values in human extracts correlated well with the sum of four major PCB congeners chemically determined in both matrices (Table 3). These results indicate that the CALUX bioassay may serve as a simple, relatively inexpensive prescreening tool to be used for exposure assessment in epidemiologic surveys, whereas

current epidemiologic surveys are almost exclusively based on chemical analysis. However, samples that induce a higher CALUX response than the limit values should undergo chemical analysis to confirm and identify the compounds responsible.

We previously evaluated the relationship between gravimetric and enzymatic lipid determinations (25). The correlation for serum lipids was strong and statistically significant ($r = 0.75$, $n = 96$, $p < 0.001$); however, gravimetric lipid determinations differ greatly between laboratories, depending on the extraction conditions and solvents used. Moreover, the extracts can contain other extractable material as well (9).

PCB-153 and PCB-180 do not possess two unsubstituted carbohydrons next to each other and are therefore highly persistent in the environment. In the same way, highly chlorinated biphenyls containing a 2,3,4-trichlorophenyl group (such as PCB-138) can resist metabolic breakdown and so accumulate rapidly in body tissues (26). We previously reported that serum concentrations of major PCB congeners are reliable predictors of follicular fluid levels (27). Except for PCB-118, the major PCB congener correlations were strong and statistically significant.

All these results were expressed on a whole-weight basis. Focusing on our recent data (Table 1), the previous associations can be confirmed. The latter correlations are even stronger and statistically significant (except for PCB-118), suggesting that expressing results on a lipid basis improves comparison of different biologic matrices.

The different mode of origin and the different lipid contents probably lead to selective accumulation in different body fluids (28). When focusing on the PCB accumulation pattern, the accumulation of individual PCBs varied only with respect to the number and positions of the chlorine substituents. In our small sample ($n = 9$), follicular fluid and serum show opposite patterns of accumulation for PCB-153 and PCB-180. These data support the findings of Schlebusch et al. (29), suggesting that the proportion of the more highly chlorinated PCBs may be increased in body compartments associated with reproduction. Certainly, different accumulation patterns can affect the toxic potential of the PCBs. If different matrices show different congener distribution, this may have an impact on the toxic outcome. These preliminary results may partly explain the somewhat higher TEQ values in follicular fluid as compared to serum TEQs.

Focusing on the comparison between the recent investigation and data from the Dutch Mother's Milk Cohort Study, Dutch women sampled 6 years earlier were considerably more exposed to dioxin-like compounds. Recent Dutch CALUX data (30) reveal TEQ levels in the same range as the recent Belgian investigation. Similar declining trends have been reported for Germany and the United Kingdom (12).

Table 2. CALUX TEQ values in Belgian female body fluids, as compared to previous CALUX data analyzed under the same analytical conditions.

|	Belgium[a]		The Netherlands[b]
	Serum ($n = 106$)	Follicular fluid ($n = 8$)	Plasma ($n = 12$)
No. above detection limit[c]	92	7	12
Mean ± SD	46.8 ± 43.8	257.9 ± 225.0	103.7 ± 51.5
Median	37.4	246.5	100.1

All values are expressed in pg TEQ/g lipid.
[a]This study (1996–1998); lipids enzymatically determined. [b]Dutch Mother's Milk Cohort Study (1990–1992); lipids gravimetrically determined. [c]Detection limit: 32 fg TEQ/well.

Table 3. Pearson correlation coefficients between PCB congener levels in body fluids (ng/g lipid) and CALUX TEQ values (pg TEQ/g lipid).

	Serum			Follicular fluid		
	No.	r	p-Value	No.	r	p-Value
PCB-118	100	0.35	< 0.001	8	-0.23	NS
PCB-138	97	0.39	< 0.001	8	0.74	0.037
PCB-153	101	0.38	< 0.001	8	0.57	NS
PCB-180	100	0.34	< 0.001	8	0.57	NS
Sum PCBs[a]	96	0.43	< 0.001	8	0.58	NS

NS, not significant.
[a]Sum PCBs is the sum of the four congeners.
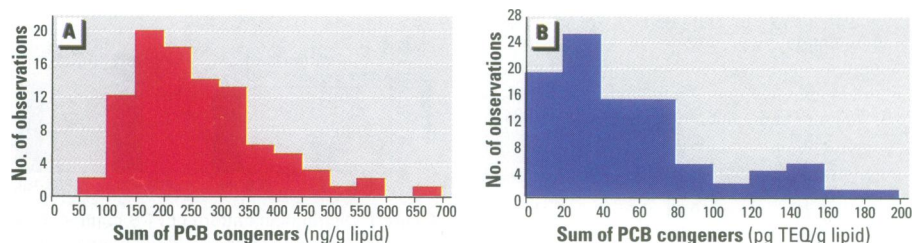
Figure 2. Distribution of (A) the sum of four PCB congeners and (B) CALUX TEQ levels in serum ($n = 106$). In (B), 14 observations are not shown due to TEQ levels below the detection limit.

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