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Determination of PCDD/Fs, PBDD/Fs and dioxin-like PCBs in human milk from mothers residing in the rural areas in Flanders, using the CALUX bioassay and GC-HRMS

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ABSTRACT

Since the CALUX (Chemically Activated LUciferase gene eXpression) bioassay is a fast and inexpensive tool for the determination of dioxin-like compounds in a large number of samples and requires only small sample volumes, the use of this technique in human biomonitoring programs provides a good alternative to GC-HRMS. In this study, a new CALUX method for the separate analysis of PCDD/Fs and dioxin-like PCBs (dl-PCBs) in small amounts of human milk samples with the new sensitive H1L7.5c1 cell line was used to analyze 84 human milk samples, collected from mothers residing in the Flemish rural communities. The geometric mean CALUX-Bioanalytical Equivalent (CALUX-BEQ) values, reported for the 84 mothers from the study area were 10.4 (95% CI: 9.4–11.4) pg CALUX-BEQ per gram lipid or 0.41 (95% CI: 0.37–0.45) pg CALUX-BEQ per gram milk for the PCDD/Fs and 1.73 (1.57–1.91) pg CALUX-BEQ per gram lipid or 0.07 (95% CI: 0.06–0.08) pg CALUX-BEQ per gram milk for the dioxin-like PCBs. Multiple regression analysis showed significant associations between PCDD/Fs and weight change after pregnancy, smoking and consumption of local eggs. One pooled human milk sample was analyzed with both CALUX and GC-HRMS. The ratio of CALUX and GC-HRMS results for this sample were respectively 1.60, 0.58 and 1.23 for the PCDD/Fs, the dl-PCBs and the sum of both fractions, when using the 2005-TEF values. Additionally, also low levels of certain brominated dioxins and furans were detected in the pooled sample with GC-HRMS.

1. Introduction

In the first Flemish Environment and Health survey performed by the Flemish Centre of Expertise on Environment and Health (FLEHS I, 2002–2006) increased concentrations of PCBs, dioxin-like substances and chlorinated pesticides were observed in cord blood of newborns and in blood of 14–15 year-old adolescents and 50–65 year-old adults living in low populated rural communities of East and West Flanders and Flemish Brabant compared to other Flemish regions [1,2]. Due to the health concern associated with increased body burdens of chlorinated POPs, a follow-up study of the pollutant levels in these rural areas was undertaken. Because POPs are mainly lipophilic, human milk was chosen to assess exposure to these compounds. Human milk is a valuable matrix for human biomonitoring of lipophilic pollutants, since it is a non-invasive sample that is available in sufficient quantities (e.g. compared to cord blood).

For the quantification of PCDD/Fs and/or dioxin-like PCBs in (human) milk samples, both GC-HRMS and CALUX bioassays are used in routine analysis. GC-HRMS analysis usually needs large amounts of human milk and is also quite expensive, while the CALUX bioassay is known as a fast, inexpensive technique that uses small sample volumes. However, so far most validated CALUX methods for quantification of PCDD/Fs and dl-PCBs in human and bovine milk samples have also used relatively large volumes. Some researchers used 60 mL milk [3–5], while others used 20 mL [6] or 10 mL milk [7–9]. Since in this Flemish human milk campaign not

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only PCDD/Fs and dioxin-like PCB were analyzed, but also other POPs (e.g. pesticides, brominated flame retardants, marker PCBs and perfluorinated compounds), the amount of human milk available for the CALUX bioassay was limited. Therefore, a new, more sensitive method needed to be developed for the separate analysis of PCDD/Fs and dioxin-like PCBs in only 5 mL of (human) milk. The aims of this study were: (1) to develop a new CALUX method for the separate analysis of PCDD/Fs and dl-PCBs in 5 mL of human milk, (2) to compare the concentrations measured in human milk samples from the rural study area to the concentrations measured in Belgian milk samples from former WHO-coordinated studies and from other national and international surveys, (3) to determine the associations between concentrations of PCDD/Fs and dl-PCBs on the one hand and personal characteristics and dietary habits on the other hand and (4) to compare the CALUX results of a pooled milk sample with the GC-HRMS data, obtained from the same sample, (5) to determine the concentration of PBDD/F congeners in human milk from the rural areas.

2. Materials and methods

2.1. Chemicals and standards

Hexane (for PCDD/Fs and PCBs, minimum 96%), acetone (Pesti-S grade, minimum 99.9%) and toluene (for PCDD/Fs and PCBs, minimum 99.8%) were purchased from Biosolve (The Netherlands). Ethyl acetate pestanal and silica gel 60 for column chromatography were purchased from Sigma-Aldrich (Germany). Sulphuric acid (95%–97%, ACS reagent), Celite 545 (0.02–0.1 mm) and DMSO were obtained from Merck (Germany). Anhydrous sodium sulphate was purchased from Boom (The Netherlands) and X-CARB from XDS (USA). The standard solution of 2,3,7,8 TCDD (50 ng mL^{-1}) was purchased from Campro Scientific (The Netherlands).

2.2. Selection, recruitment of participants and sample collection

The participants were recruited from nine maternities in East and West Flanders. Since the POP levels were meant to be compared with the Belgian results of the WHO human milk surveys from 1987–1988, 1992–1993, 2000–2003 and 2005–2006 [10], the selected mothers had to meet the WHO inclusion criteria for age, parity and single birth and residence time of at least 5 years in the study area. However, because the recruitment turned out to be rather difficult, the selection criteria were broadened [11]. During a period of 14 months (May 2009 until end of June 2010), finally, a total of 84 mothers (30.6% of the selected mothers) participated in the study. At home, the mothers collected the milk two to eight weeks after delivery. At least 50 mL of milk samples had to be collected, after and/or during nursing. The samples were stored at −20 °C until analysis.

After signing the informed consent, the mothers were asked to complete a questionnaire for information about residence during the last 5 years, date and place of birth, mother's age, weight and length, dietary habits and occupation, smoking and alcohol consumption, fertility and health data, exposure to pollutants indoor or in the workplace, socio-economic status and perception of environmental problems. More details about the personal characteristics of the participants were described by Croes et al. [11].

The study design was approved by the medical-ethical committee of the University of Antwerp on 10th of July 2009.

2.3. Analytical procedure for quantification of PCDD/Fs and dl-PCBs

PCDD/Fs and dl-PCBs were analyzed in all 84 individual samples using the CALUX bioassay. From each of the 84 individual samples, 10 mL human milk of the initial sample was taken to compose a pooled sample. This pooled sample was used to follow up the time trend of PCDD/F and dl-PCB concentrations that were measured during the former WHO-coordinated human milk surveys [10]. GC-HRMS analysis of pollutants in the pooled sample was done by the WHO reference lab (State Institute for Chemical and Veterinary Analysis of Food (CVUA), Freiburg, Germany), according to the method described by Malisch and van Leeuwen [12], Kotz et al. [13] and Hui et al. [14]. CALUX analysis was performed by the Department of Analytical and Environmental Chemistry at the Free University of Brussels (VUB, Brussels, Belgium). For the determination of dioxin-like compounds in human milk samples, the same protocol as for extraction and clean up of human serum samples was used [15], except for a higher amount of acid silica due to the higher lipid content of human breast milk. Briefly, 5 mL human milk samples were extracted with an acetone/hexane mixture and filtered upon a pre-conditioned celite column. After extraction, the amount of fat was weighted and the extract was redissolved in 5 mL hexane and cleaned up on a pre-conditioned multi-layer silica column coupled in series with a carbon column. The silica gel column (25 mL) was filled from bottom to top with glass wool, 1.9 g (1.3 cm^3) sodium sulphate, 6.0 g (2×4.3 cm^3) of 33% (w/w) sulphuric acid silica gel and 1.9 g (1.3 cm^3) sodium sulphate. The carbon column (10 mL) was filled with glass wool, 0.7 g (0.5 cm^3) sodium sulphate, 0.34 g (1 cm^3) X-CARB and 0.7 g (0.5 cm^3) sodium sulphate. The dioxin-like PCBs and the PCDD/Fs were eluted separately from the carbon column and redissolved in a defined volume hexane (1.5 mL and 2 mL for the PCB and PCDD/F fractions, respectively). Concentration–response analysis using pooled milk sample extracts allowed determination of an optimal dilution factor to facilitate screening analysis and to minimize sample volumes needed for analysis. For the dl-PCB fraction a final dilution factor of 1.5 was used, while dilution factors 2.5 and 4 were used for the PCDD/F fraction. The target compounds were analyzed using the enhanced recombinant mouse hepatoma CALUX cell line (H1L7.5c1) which had been stably transfected with an Ah receptor-responsive firefly luciferase reporter gene plasmid (pGudLuc7.5) that contains 20 dioxin-responsive elements [16,17]. The results were expressed as CALUX bioanalytical equivalents (BEQs), using the inverse prediction method on a Hill-shaped TCDD calibration curve. The final result was expressed in pg BEQ per gram fat and in pg BEQ per gram milk.

2.4. Statistics

Means, medians, ranges and geometric means were calculated using SAS 9.2 and Statistica 10.0. The geometric means were obtained after back transformation of the mean values of the Ln transformed variables. To determine the factors that influence the PCDD/F and dl-PCB levels in the human milk samples, univariate regression relationships were first calculated. Covariates with a p-value lower than 0.25 were entered in a multiple regression analysis (stepwise selection). Only variables with a p-value less than 0.10 were retained in the multiple regression models. For samples below the limit of quantification (LOQ), half of the LOQ was used. Age, body mass index (BMI) and smoking habits were included as fixed confounders for PCDD/Fs and dl-PCBs in the multiple regression models. For age and BMI, a continuous variable was used as confounding factor, while for smoking the categorical variable current-, ex- or non smoker was used in regression analysis. Univariate and multiple regression analyses were performed on Ln-transformed PCDD/F and dl-PCB values, expressed in pg BEQ per gram lipid weight.