CALUX DETERMINATION OF PCDD/F'S AND DIOXIN-LIKE PCB'S IN SMALL AMOUNTS OF HUMAN SERUM WITH THE H1L7.5C1 CELL LINE: ANALYSIS OF SERUM SAMPLES FROM THE FLEMISH ENVIRONMENT AND HEALTH STUDY (FLEHS II)

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Introduction

Although emissions of PCDD/Fs and PCBs have decreased during recent years, these compounds are still environmental pollutants of concern: 1) since PCDD/Fs and dioxin-like PCBs (dl-PCBs) are persistent in the environment, accumulate in fat tissue and have hormone disrupting properties and 2) because emissions in certain locations in Flanders are still high. Therefore, it is important to include these compounds as biomarkers of exposure in human biomonitoring programs. In 2007, a second cycle of the Flemish Human Biomonitoring program (FLEHS II) started. More than 40 biomarkers of exposure and 10 effect markers were measured in 650 samples, recruited from 14-15 year-old adolescents (n=200), 20-40 year old-adults (n=200) and mother-newborn pairs (n=250). Since only a small amount of serum was available for the PCDD/F and dl-PCB determination, GC-HRMS analysis was not possible. The CALUX bioassay provided a good alternative, since it requires only 5 mL of serum. This publication presents an optimized method for the separate analysis of PCDD/Fs and dl-PCBs in human serum with the newly developed and more sensitive third generation CALUX (H1L7.5c1) cell line.

Materials and methods

The extraction and clean up procedure for the analysis of PCDD/Fs and dl-PCBs was based on the protocol used by Schroijen et al. (2006)[1]. The cell line used in the bioassay was the sensitive H1L7.5c1 recombinant mouse hepatoma cell line, stably transfected with pGudLuc 7.5 and containing 5 dioxin responsive domains (5 DRDs)[2,3]. Cell treatment and measurement were based on the protocols described by Windal et al. (2005)[4]. A four parameter Hill-function was used to fit a sigmoid curve through the standard solutions. The measured luminescence in relative light units (RLU) of an unknown sample was converted into a bioassay toxic equivalency value (CALUX-BEQ) by comparison of the response of the sample to the sigmoid dose-response curve obtained with 2,3,7,8-TCDD standards. Three quality control (QC) solutions (i.e. a standard solution of TCDD corresponding to a RLU induction of around 50%) and 3 DMSO blanks were added in duplicate to every 96-well plate as an internal control.

Results and discussion:

In this study, 5 mL serum samples obtained from 173 14-15 year-old adolescents of the general population, recruited via the school system in Flanders, were analyzed for PCDD/Fs and dl-PCBs with a new sensitive CALUX mouse hepatoma cell line. This H1L7.5c1 cell line was specially designed to analyze low concentrations in small sample volumes. With the less sensitive H1L6.1c3 cell line, which was commonly used in previous biomonitoring studies and food/feed analysis, only a single-point analysis of the whole extract was often used and it was not possible to measure the dl-PCB fraction, since most samples were below the quantification limit. With the H1L7.5c1 cell line, 98.8% of the dl-PCBs could be quantified and multiple dilutions of the PCDD/F extract could be analyzed to optimize the working range.

Before starting the routine analysis, first, full dose-response dilution curves using pooled serum sample extracts were established to allow the determination of an optimal dilution factor to facilitate screening analysis and to minimize sample volumes needed for analysis. Figure 1 shows a full dose-response dilution curve for the PCDD/F fraction, measured with the H1L7.5c1 cell line.

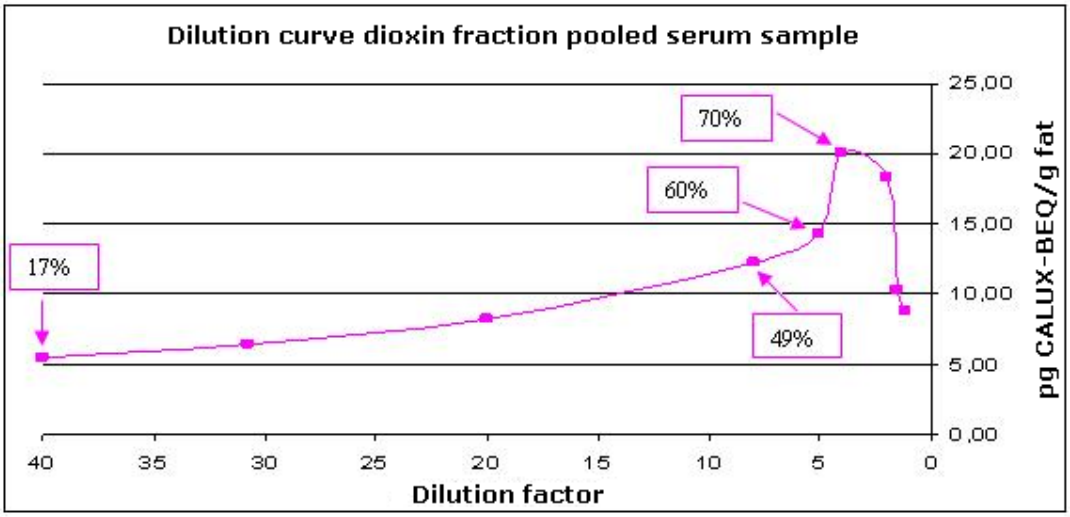

Figure 1: Full dose-response curve for the PCDD/F fraction from pooled serum samples, measured with the H1L7.5c1 cell line. The percentage induction (RLUs relative to TCDD) is shown at the positions indicated by the arrows.

From this figure it is clear that the optimum dilution factor (df) is in the range of 4, 5 and 8, with induction levels between 70 and 49 %. When incubating cells with more diluted samples (df 20 to 40), the induction levels were rather low and too many samples would be below the quantification limit (LOQ). When dosing more concentrated samples (df 1.2, 1.5 or 2), the % RLU induction was again lower than the curve maximum (past the top of the curve) and the sample extracts were in the so called "toxic range" of the calibration curve. Since no cell death was observed in these cells, there were probably interfering compounds from the serum matrix that suppressed the CALUX signal and/or the induction response. Sample dilution factors of 5 and 2.4 were selected for routine analysis of respectively the PCDD/Fs and dl-PCBs, since these dilutions induced luciferase activity close to the EC50 value (~ 438.6 fg/well) of the 2,3,7,8-TCDD standard curve.

The validation studies showed that repeatability and within-lab reproducibility for the QC standard were < 15%. A long-term within-lab reproducibility (RSD_{RW}) of 25% for the PCDD/F fraction and 41% for the dl-PCB fraction for the analysis of pooled serum samples, expressed as pg BEQ/g fat, was determined. CALUX recoveries of the spiked procedural blanks were within the accepted in-house limits of 80-120% for both fractions. The LOQ was 30.3 pg BEQ/g fat for the PCDD/Fs and 14.5 pg BEQ/g fat for the dl-PCBs, taking into account a mean amount of fat in the serum sample of 0.0165 g for the adolescents and a dilution factor of 5 for the PCDD/Fs and 2.4 for the dl-PCBs. The GC-HRMS recovery of a C13-spiked pooled serum sample was between 60-90 % for all PCDD/F congeners and between 67-82 % for the non-ortho PCBs. Also, an adequate separation between both fractions was found: a maximum of 13 % of PCDD/F congeners was found in the PCB fraction and maximum 14% of PCB congeners were found in the PCDD/F fraction. The CALUX/GC-HRMS ratio for a pooled serum sample was respectively 2.0 and 1.4 for the PCDD/Fs and the dl-PCBs indicating the presence of additional AhR active compounds (Table 2).

After adjustment for covariates such as age, sex, BMI, smoking and amount of blood fat, the geometric mean in the total study group (n=173) was 110 (95% CI: 104-116) pg BEQ/g fat for the PCDD/Fs and 32.7 (30.7-34.7) pg BEQ/g fat for the dl-PCBs. Both biomarkers were higher in boys than in girls (ANOVA: $p<0.001$). The PCDD/F CALUX-BEQ value increased with a lower education level ($p=0.02$) and was significantly higher in subjects consuming self-caught fish ($p=0.02$). For dl-PCBs only a non-significant increasing trend was seen for some food factors such as consuming self-caught fish ($p=0.12$) and local eggs ($p=0.17$) and being breastfed as newborn ($p=0.07$).

These CALUX-BEQ values will be used as a reference value for Flanders. The PCDD/F values were relatively high compared to most values from human biomonitoring studies that have been reported in literature (Table 1), but it is not always clear which sample analysis protocol was followed in the other studies. In biomonitoring studies often different techniques are used (CALUX rat cells, CALUX mouse cells, GC-HRMS and analysis with or without separation of PCDD/Fs and dioxin-like PCBs), which makes clear interpretation and comparison of the results difficult. Also differences between clean up methods, the used dilution factor employed, different methods for quantification using CALUX standard curves[5] and the choice of TEF/REP values for quantification with GC-HRMS, etc. have an important influence on the final result. Therefore, it is only possible to compare different studies if exactly the same protocols and dilutions are used or, otherwise, if a correlation between the two methods has been established. The latter can be done by analyzing the same pooled sample(s), with a concentration(s) in the expected range of the unknown samples, multiple times with both methods.

Reference	Country	Period	Population	N	Calculation	Unit	Value	Method
CALUX in pg TEQ/g fat								
This Study	Flanders	2008-09	Students (14-15 years old), general population	173	GM (95%CI)	pg CALUX-BEQ/g fat	110 (104-116)	UDC-CALUX, H1L7.5c1 PCDD/F
This Study	Flanders	2008-09	Students (14-15 years old), general population	172	GM (95%CI)	pg CALUX-BEQ/g fat	32.7 (30.7-34.7)	UDC-CALUX H1L7.5c1, dl-PCB
Van Wouwe et al., 2004	Belgium	2000	Adults, men and women	341	GM	pg CALUX TEQ/g fat	41.8	XDS-CALUX, H1L6.1c2 PCDD/F
	Belgium	2000	Adults, men and women	341	GM	pg WHO-TEQ/g fat	25.7	GC-HRMS, PCDD/F
Long et al., 2006	Greenland	2002-2004	Adults, men	75	median	pg CALUX TEQ/g fat	197	UCD-CALUX, Hepa1.12cR
	Poland	2002	Adults, men	99	median	pg CALUX TEQ/g fat	312	UCD-CALUX, Hepa1.12cR
	Sweden	2002	Adults, men	78	median	pg CALUX TEQ/g fat	428	UCD-CALUX, Hepa1.12cR
	Ukraine	2002	Adults, men	86	median	pg CALUX TEQ/g fat	337	UCD-CALUX, Hepa1.12cR
Koppen et al., 2001	Flanders, Peer	1999	Adults, women, 50-65 years old	22	Mean (SD)	pg CALUX TEQ/g fat	37.2 (13.1)	BDS-CALUX, sum PCDD/F and dl-PCB
	Flanders, Antwerp	1999	Adults, women, 50-65 years old	25	Mean (SD)	pg CALUX TEQ/g fat	35.0 (16.5)	BDS-CALUX, sum PCDD/F and dl-PCB
	Flanders, Peer	1999	Adults, women, 50-65 years old	22	GM (95%CI)	pg WHO-TEQ/g fat	70.9 (65.3-76.9)	GC-HRMS, sum PCDD/F and dl-PCB
	Flanders, Antwerp	1999	Adults, women, 50-65 years old	25	GM (95%CI)	pg WHO-TEQ/g fat	78.9 (72.7-85.6)	GC-HRMS, sum PCDD/F and dl-PCB
Kayama et al., 2002	Japan	2002	Female farmers, 55,5 years old (average)	1407	Mean (SD)	pg CALUX TEQ/g fat	32.3 (12.1)	XDS-CALUX, PCDD/F
Todaka et al.,2010	Japan	2002-2005	Mothers	119	Mean (SD)	pg WHO-TEQ/g fat	11(4.2) PCDD/F 5.5 (2.5) dl-PCB	GC-HRMS
Wittsiepe et al., 2007	Germany	2000-2003	Pregant women, 19-42 years old	169	Mean	pg WHO-TEQ/g fat	16.79 PCDD/F 11.57 dl-PCB	GC-HRMS
Burns et al., 2009	Russia	2003-2005	Children 8-9 years old	482	Median	pg WHO-TEQ/g fat	21.1	GC-HRMS
Ayotte et al., 2005	Canada	na	Adults, men and women, 25-75 years old	40	Median (min-max)	pg CALUX TEQ/g fat	102 (37-287)	BDS-CALUX, sum PCDD/F and dl-PCB
Warner et al.,2005	Italy	1999	Women, 20-49 years old	22	Mean (min-max)	pg CALUX TEQ/g fat	30.8 (1.6-67.3)	XDS-CALUX, PCDD/F

Table 1: Literature overview for the PCDD/F concentrations from human biomonitoring studies in different countries

	PCDD/Fs	PCBs	Sum PCDD/Fs and dl-PCBs
GC-HRMS (pg WHO-TEQ/g fat)	41.7	14.7	56.4
CALUX (pg BEQ/g fat)	83.3 (n=51)	20.0 (n=35)	103.3
Ratio CALUX/GC-HRMS	2.0	1.4	1.8

Table 2: Ratio between CALUX and GC-HRMS for the PCDD/F and PCB fraction of a pooled serum sample

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