USING THE CALUX BIOASSAY FOR SCREENING AND DETERMINATION OF DIOXIN-LIKE COMPOUNDS IN HUMAN MILK

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Introduction

Dioxins, PCBs and other related compounds constitute a group of persistent environmental contaminants exhibiting a broad spectrum of biological and toxic effects. Due to their lipophilic character and metabolic stability they accumulate in the food chain and biological matrices including human adipose tissue, blood and milk. The vulnerability of the food chain has been illustrated by some of the recent incidents where feeding stuffs have been contaminated with dioxins or PCBs. To prevent the consumer from unacceptably high exposure of these compounds, The Commission of the European Communities is considering setting maximum limits for dioxins in food and feeding stuffs. In order to monitor the levels of dioxin-like compounds in food, feeding stuffs and human tissues there is a need for screening methods with a high sample throughput. The cell-based bioassay CALUX (Chemical Activated Luciferase Expression) is a reporter gene assay that determines the total dioxin-like activity in a sample[1].
The objective of the present study was to validate the CALUX bioassay as a screening method for human milk samples by 1) testing the additivity of standard dioxin-like compounds in a mixture 2) determine the variations for sample preparation and clean-up procedure and 3) determine the total TEQ in human milk samples by comparing the data obtained in CALUX with GC/MS data.

Methods and Materials

CALUX bioassay

Rat H4IIE hepatoma cells stably transfected with an AhR-controlled luciferase reporter gene plasmid (pGudLuc1.1) were a generous gift from Dr. Abraham Brouwer, Vrije University, Amsterdam, The Netherlands[1]. The cells were grown in 96-well microtiter plates containing 100 µl MEMα culture medium supplemented with 5% fetal bovine serum (BioWhittaker) and 1% penicillin/streptomycin. The density of the cells was 2.2 * 10^4 cells/well. After an incubation period of 24 hours (37°C with humidified atmosphere of 5% CO_2/air) the cells reached 90-100% confluency. The test compounds were added in a final concentration of 0.4% DMSO and 1% FBS. 2,3,7,8-TCDD standard series from 0.3 to 1000 pM were included on each microtiter plate. All standards and samples were tested in triplicate. After 20-24 hours of exposure the cells were washed with PBS and lysed with 25 µl cell lysis reagent. Aliqouts of 10 µl extract were transferred to a white microtiter plate and the light production was measured using a luminometer. TCDD-equivalents (TEQ) were calculated for each sample. Standard solutions were tested for cytotoxicity towards the cells using the AlamarBlue assay®.

Study of interaction between dioxins and PCBs in human milk

Initially seven standards comprising the major dioxin-like compounds in human milk (2,3,7,8-TCDD; 1,2,3,7,8-PeCDD; 1,2,3,6,7,8-HxCDD; 2,3,4,7,8-PeCDF; PCB 118; PCB 126; PCB 156)

and a mixture of these were tested. Each congener in the mixture was divided relative to its concentration in an average human milk sample. The concentration-response curves for the seven compounds and the mixture were tested for additivity by the method of isoboles.

Human milk samples
Sixteen human milk samples were collected in Denmark (four in 1993 and twelve in 1999) among primiparae between 25-29 years. The milk was sampled between 3-8 weeks after delivery and represents the average Danish population.

Sample preparation
The clean-up procedure for fish oil and human milk samples (1 g fat) was performed on silica gel coated with 33% H_2SO_4. The column was eluted with n-pentane and after solvent removal by evaporation; the residue was dissolved in 30 µl DMSO. One fish oil sample and sixteen samples of human milk were extracted. Total TEQ-values for the human milk samples were determined in the CALUX bioassay and compared to TEQ obtained from GC/MS analysis (WHO-TEFs). Five extracts of human milk samples were further subfractionated by HPLC into three fractions containing dioxins/furans, non-ortho PCBs and mono/di-ortho PCBs. The sum of TEQ-values in the subfractions was compared to TEQ-values in the whole sample extracts.

GC/MS-determination
Before fat extraction and clean up, samples were spiked with a mixture of carbon-13 labelled PCB, PCDD and PCDF internal standards. The final extracts were dissolved in 20 µl toluene and 2.5 µl was injected by a Fisons AS800 autosampler into a Fisons 8065 gas chromatograph interfaced to a Micromass AutoSpec Ultima high resolution mass spectrometer.

Statistics
The TCDD standard curve was fitted using linear regression (SigmaPlot 5.0, SPSS) for concentrations between 0.6-10 pM. Unknown samples were interpolated on this curve calculating a TEQ value for each sample.

Results and Discussion
The seven compounds, comprising the major dioxin-like compounds in human milk, and a mixture of these showed a concentration-dependent increase in luciferase activity in the CALUX bioassay (Fig 1). As expected, TCDD was the most potent dioxin-like compound giving rise to a response around 0.6 pM and reaching plateau around 100 pM. All standards had a comparable maximum response although PCB118 showed cytotoxicity at concentrations above 10 µM. When comparing the data for each compound and the mixture by the method of isoboles (concentration addition), significant additive responses were observed at the two relative effect levels (10 and 40). This indicates that no interaction between these dioxin-like congeners takes place at the receptor level. Except for the weaker agonist PCB 118, there was a good correlation between WHO-TEFs and REPs (relative potencies) obtained in the CALUX bioassay (Table 1). A similar finding was reported by Bovee et al. 1998[2].

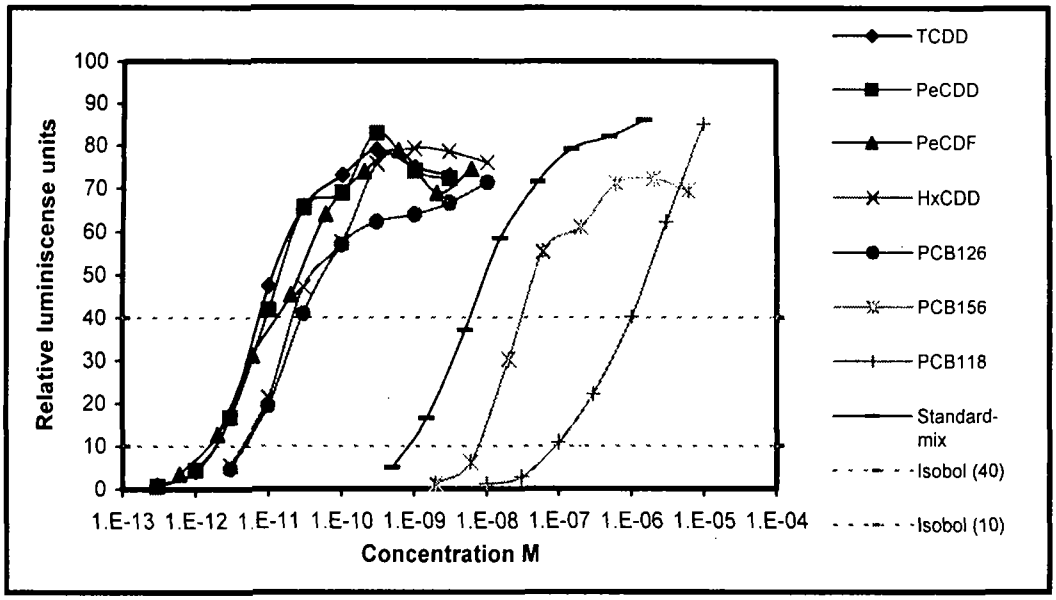

Fig 1. Activation of the Ah-receptor by dioxin-like compounds in the CALUX bioassay.

Compound		EC50 (pM)	CALUX-REP	WHO-TEF
2,3,7,8-TCDD		8	1	1
1,2,3,7,8-PeCDD		11	0.79	1
2,3,4,7,8-PeCDF		16	0.51	0.5
1,2,3,6,7,8-HxCDD		23	0.36	0.1
3, 3', 4, 4', 5-PeCB	PCB 126	30	0.28	0.1
2, 3, 3', 4, 4', 5-HxCB	PCB 156	2.8×10^{4}	3.0×10^{-4}	5.0×10^{-4}
2, 3', 4, 4', 5-PeCB	PCB 118	1.2×10^{5}	6.8×10^{-6}	1.0×10^{-4}

Table 1. Relative Potencies (REPs) for induction of luciferase in H4IIE rat hepatoma cells of 7 selected dioxin-like compounds compared with their Toxic Equivalency Factors (WHO-TEFs).

Testing six separate extractions of one fish oil sample in the CALUX bioassay showed an average of 10.0 pg TEQ/g fat and a intra-assay variation of 3%. This indicates a high repeatability of the whole procedure from the extractions throughout the cell assay. The limit of detection for a sample was 1.4 pg TEQ/g fat.

All sixteen human milk samples showed higher TEQ-values when tested in the CALUX bioassay than determined with GC/MS (Table 2). Three independent experiments with each total sample showed an inter-assay variation between 6-49%. Five samples were fractionated by HPLC into groups of dioxins/furans, non-ortho PCBs and mono- and diortho PCBs and the results showed comparable TEQ-values for the total sample and the sum of dioxin and PCB fractions. The higher TEQ-values obtained in CALUX indicate either deviations between WHO-TEF and CALUX-REP or that so far unidentified compounds contribute to the total dioxin-like activity. These potential unidentified compounds seem to be present in the fractions that normally contain dioxins/furans and PCBs.

This study demonstrates the CALUX bioassay as a valuable screening method for monitoring human exposure to dioxin-like compounds. Recently the assay has been validated for specific food

samples and tested towards screening of serum samples[3,4], but additional studies including other food matrices and human samples are necessary for a full validation of the CALUX bioassay.

Human milk 16 samples					
Sample	GC/MS Determination pg TEQ/g fat	CALUX Total sample pg TEQ/g fat	CV % Total sample	CALUX Sum of fractions pg TEQ/g fat	CV % Sum of fractions
1	36.5	39.6	26	38.4	14
2	35.8	36.3	20	35.5	14
3	36.2	55.8	7	67.1	6
4	36.2	39.2	9	46.8	11
5	19.4*	34.2	15		
6	16.9*	20.5	13		
7	22.6*	50.1	12		
8	19.8*	22.7	11		
9	31.5*	40.8	10		
10	17.6*	24.2	22		
11	14.6*	22.2	15	30.6	8
12	16.1*	29.7	11		
13	13.4*	27.2	12		
14	22.3*	34.7	6		
15	14.5*	24.2	49		
16	38.5*	42.3	31		

Table 2. Mean TEQ-levels in 16 human milk samples determined in CALUX bioassay in three independent experiments. Determined by GC/MS. (*not including mono-ortho dioxin-like PCBs)

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References

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