CALUX™ RESULTS CORRELATE WITH GC/MS/MS DATA FROM KAZAKHSTAN BREAST MILK SAMPLES

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Introduction

In 1994, in the first comprehensive investigation of persistent organochlorine contaminants in a country of the former Soviet Union, we measured congener-specific levels of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), as well as 19 organochlorine pesticides (OC) in breast milk samples collected using the WHO protocol from first-time mothers ("primiparae") living in southern Kazakhstan[1-3]. High levels (up to 80 pg/g fat) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were found in breast milk samples from villages in a cotton-growing region in southern Kazakhstan, with TCDD contributing 70-80% of the I-TEQ[1,2]. In these samples, the I-TEQ arose almost exclusively from two congeners, TCDD and the pentachlorodibenzo-p-dioxin (PeCDD). A follow-up study in 1997 measured levels of PCDDs/PCDFs in samples from the region's cotton-growing State Farms[4]. As part of our continuing characterization of this TCDD contamination, we investigated the effectiveness of the CALUX™ assay to screen and identify breast milk samples with high contaminant levels. We report the results of correlation studies on CALUX™ and GC/MS/MS data using these samples, where two dioxin congeners are responsible for most of the I-TEQ.

Materials and Methods

Study design. Twenty-one breast milk samples and their organic extracts were used in the correlation studies. The samples were collected in 1997 using the WHO/EURO, protocol for breast milk sample collection, in which donors were healthy primiparae with healthy infants 2-8 weeks of age[5]. The procedures followed in these exposure assessment studies (design, exposure assessment questionnaire, informed consent, PCDD/PCDF target analytes, analytical methods, and statistical analysis) are described in detail elsewhere[1,2].

Split samples of breast milk were analyzed by quadrupole ion storage tandem mass spectrometry (GC/MS/MS) and by the CALUX™ assay. Results from the two methods were compared using statistical tests.

Analytical methods. Breast milk samples (100 mL) were collected from donors in chemically clean sample jars with teflon-lined caps, frozen immediately, and stored at -20°C until analysis. Samples were thawed, shaken, and weighed, and a 5 gram aliquot was removed for the CALUX™ assay ("milk aliquot"). The remainder was extracted in ethanol:hexane:diethyl ether

(2:1:1), and a 10% aliquot was set aside for CALUX™ assay ("extract aliquot"). The remainder of the extract was evaporated to dryness, spiked with ^{13}C standards, cleaned-up, and the residue analyzed by GC/MS/MS

GC/MS/MS. Extracts of breast milk samples were spiked with 15 $^{13}C_{12}$-labeled PCDD/PCDF standards and analyzed for congener-specific PCDD/PCDFs in a U.S. Food and Drug Administration (USFDA) laboratory using GC/MS/MS[7]. Lipids were determined gravimetrically, and residue levels were expressed as pg/g milk lipid. I-TEQs for PCDDs/PCDFs were calculated using both old and new TEF values[8,9].

CALUX™ ***Assay.*** Xenobiotic Detection Systems, Inc. (XDS), has a patented genetically engineered cell line which contains the firefly luciferase gene under trans-activational control of the aryl hydrocarbon receptor[10]. The cell line can be used for the detection and relative quantification of PCDDs, PCDFs, and co-planar PCBs when used with our patent pending sample process procedure[11]. The assay using this cell line is called the Chemical-Activated Luciferase expression, or CALUX™, assay

Using the CALUX™ assay, breast milk samples ("milk aliquots") and the set of organic extracts ("milk extracts"), stored at –20° C, were analyzed for total TEQ activity as expressed by the reporter gene. The "milk aliquots" (5 ml) were transferred to hexane-rinsed glass vials with a PTFE-lined cap and were shaken and extracted three times with an acetone/hexane mixture. The three extracts were pooled and evaporated to dryness under nitrogen, and the remaining residue was weighed to determine organic extractibles ("milk lipid"). The residues of "milk aliquots" and "milk extracts" were re-suspended in hexane and cleaned up for the bioassay using our proprietary, patent=pending clean-up procedure[11].

The sample extracts in DMSO were suspended in cell culture medium, just prior to dosing on monolayers of H1.L1.6 mouse hepatoma cells that were grown in 96-well culture plates. In addition to the dilution of samples, a standard curve of TCDD-concentrations was assayed (128.8, 64.4, 32.2, 16.1, 8.0, 4.0, 2.0, 1.0, 0.5, and 0.1 ppt of TCDD). The plates were incubated for optimal induction of luciferase activity in a humidified CO_2 incubator. After the incubation, the media was removed and the cells were microscopically observed for viability. The luciferase activity was quantified using the substrate kit of Promega™ (Madison, WI).

Statistical Analysis. Analytical data were stored in EXCEL 97 (Microsoft, Redmond, WA). All statistical analyses were conducted in STATA 6.0 (Stata Corp, College Station, TX). Spearman Rank Correlation was used to assess the strength of the linear association between results from the GC/MS/MS and CALUX™ analytical methods.

Results and Discussion

From the GC/MS/MS analysis of milk samples, the I-TEQs (pg/g fat) were calculated using both the old and new TEFs (PeCDD TEFs of 0.5 vs 1.0, respectively). The CALUX™ assay was run on the provided milk (n = 13) and milk extract samples (n=21). For the 8 extract samples with no milk samples, CALUX™ results were predicted using regression analysis of CALUX™ data

from the 13 milk and milk extract samples after log transformation. Thus, a CALUX™ milk sample data set with 21 observations was assembled from 13 measured and 8 extrapolated values.

Correlation Analysis. The relationship between the GC/MS/MS and CALUX™ results was examined by an extensive correlation analysis. For the milk samples, the linear relationship between GC/MS/MS and CALUX™ results was stronger when new, rather than old, TEF values were used, and when all PCDD/PCDF congeners were included in the calculation of the I-TEQ (Table 1).

Table 1: Correlation Coefficients (Spearman)* for GC/MS/MS and CALUX™

		CALUX™	
		Milk (original n=13)	Milk 13 + 8 predicted (n=21)
GC/MS/MS	I-TEQ new TEF (n=21)	0.7418	0.7819
	I-TEQ old TEF (n=21)	0.6823	0.7379
	TCDD+PeCDD only, new TEF (n=21)	0.7363	0.7532
	TCDD+PeCDD only, old TEF (n=21)	0.6648	0.6857
	TCDD only (n=21)	0.5942	0.6329

* test of $\rho = 0$, all p values <0.02.

Sensitivity, Specificity, Predictive Values of the* CALUX™ *assay. Based upon the rankings of CALUX™ results for the 21 milk samples (13 measured and 8 predicted), four cutoff points were selected for analysis of specificity, sensitivity, and predictive values of the CALUX™ assay: 25, 38.12 (CALUX™ median TEQ), 45, and 60 pg/g fat. Results are summarized in Table 2. The median cutoff provides the best balance of specificity and sensitivity.

Table 2: Sensitivity, Specificity, Predictive Values of CALUX™ Assay

	Cutoff Point			
Estimate of Measure	25 pg/g fat	38.12 pg/g fat (CALUX™ median)	45 pg/g fat	60 pg/g fat
Sensitivity	0.94	0.78	0.50	0.33
Specificity	0.20	0.75	0.92	1.00
Predictive Value (positive test result)	0.79	0.70	0.80	1.00
Predictive Value (negative test result)	0.50	0.82	0.75	0.79

Definitions:
Sensitivity: P(CALUX™>=cutoff | GC/MS/MS >=cutoff)
Specificity: P(CALUX™<cutoff | GC/MS/MS<cutoff)
Predictive value (positive test result): P(GC/MS/MS>=cutoff | CALUX™>=cutoff)
Predictive value (negative test result): P(GC/MS/MS<cutoff | CALUX™<cutoff)

TEQs measured in human milk using the CALUX™ method correlated with I-TEQs calculated using GC/MS/MS data when TEQs were as low as 1 pg/g wet weight. The correlation of CALUX™ with GC/MS/MS was strengthened when using a PeCDD TEF = 1.0 instead of the older TEF (0.5). This small data set suggests that CALUX™ is useful as a screening assay, and warrants further study.

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