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EXPERIENCES FROM AN INTERNATIONAL INTERCALIBRATION OF DIOXIN-LIKE COMPOUNDS IN COD LIVER USING BIOASSAYS

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Introduction

This intercalibration study is an international exercise to validate the use of bioassays to determine levels of dioxin-like compounds in food. It was open for academic, regulatory and commercial laboratories. A full report of the intercalibration, which also includes bioassay analysis of fly ash and fly ash extract, is available from the authors [1]. The study, performed during 2002 attracted considerable interest, and initially we received registrations from 18 laboratories world-wide. The objectives of the study were:

- to estimate the agreement between bioassay results and GC/MS-determined levels of PCDDs, PCDFs and coplanar PCBs in a cod liver sample
- to study differences in sensitivity between different bioassays
- to assess interlaboratory variation for similar bioassays

The study took place from March to October 2002 and preliminary results were presented at a closed meeting during the Dioxin 2002 conference in Barcelona, Spain. The registrations were from bioassay laboratories in Norway, Denmark, Germany, The Netherlands, Belgium, France, The Czech Republic, USA, and Japan. After the final reporting deadline, 14 laboratories had reported their results. The participating laboratories were the Aquatic Toxicology Laboratory, Michigan State University; Scientific Institute Of Public Health, Belgium; Centre of Analysis of Residue in Traces, University of Liege; Vito-Environmental toxicology, Belgium; Xenobiotic Detection Systems, USA; HIYOSHI Corporation, Japan; Aquatic toxicology, Department of Zoology, University of Heidelberg; Institute of Food Safety and Toxicology, Denmark; Department of Chemistry and Toxicology, Veterinary Research Institute, Czech Republic; Norwegian Institute for Air Research, Norway; Junior Research Group of Molecular Animal Cell Toxicology, UFZ Centre for Environmental Research, Germany; Institute of Environmental Medicine, Karolinska Institute, Sweden; GSF-Research Center For Environment And Health, Institute For Ecological Chemistry, Germany and Biodetection Systems, The Netherlands.

Methods and Materials

Liver (1.5 kg) from cod purchased in Oslo in December 2001. The liver was homogenised thoroughly and frozen. Before shipment, the homogenate was thawed, homogenised once more and aliquots of 15 g were placed in scintillation glass vials. The frozen samples for each laboratory were placed in a metal container that was filled with adsorbent material in case of leakage. The samples were distributed using an international courier service and arrived unharmed to the laboratories within 2-3 days. The laboratories were instructed to use their own extraction and clean-up procedures for the samples. They were asked to perform three tests of each processed extract from the cod liver.

The cod liver samples were also sent to two well-renowned laboratories for chemical analysis (HRGC/HRMS) of PCDD/Fs and non- and mono-ortho PCBs. The TEQ concentrations were calculated using human WHO-TEFs.

Below is a short description of the bioassays.

DR-CALUX
This is a reporter gene-based bioassay using the luciferase gene under control of DRE sequences. It is based on the H4IIE GudLuc1.1 rat hepatoma cell line, which has been stably transfected with the plasmid pGudLuc1.1 [2]. The luciferase induction is measured after culturing and is correlated to TEQ exposure. The culturing time varied between 22 and 24 hours.

H4IIE-luc
This is a recombinant cell line containing a luciferase reporter gene under control of DRE sequences. It is based on the H4IIE-luc rat hepatoma cell line, which has been stably transfected with the plasmid pGudLuc1.1 [3]. The luciferase induction is measured after culturing and is correlated to TEQ exposure. The culturing time was 72 hours.

CALUX and DIPS-CALUX
This is also a reporter gene-based bioassay using the luciferase gene under control of DRE sequences. It is based on the Hepa 1 mouse hepatoma cell line that has been stably transfected with a plasmid containing the luciferase gene under control of DRE sequences [3].
The abbreviation DIPS stands for dioxin/furan and PCB specific, which according to the participating laboratories is a selective clean-up method to isolate PCDD/Fs from PCBs. After in vitro cell culturing, luciferase induction is correlated to TEQ exposure. The culturing time was 20-24 hours.

EROD-micro-bioassay
This is a cell-based bioassay using induction of EROD activity. It is based on the cell line H4IIEC/T3, which was originally isolated from a rat hepatoma [4]. After cell culturing, EROD induction is correlated to TEQ exposure. Culturing times were 24 and 72 hours.

MH1C1 EROD assay
This is a cell-based bioassay using induction of EROD activity. It is based on the cell line MH1C1, which was originally isolated from a rat hepatoma [5]. After cell culturing, EROD induction is correlated to TEQ exposure. The culturing time was 24 hours.

Results and Discussion
Chemical analysis
The concentrations of PCDDs/Fs, non- and mono-ortho PCBs, expressed as WHO-TEQs were 27 pg/g wet weight for the cod liver.

Bioassay analysis
The cod liver was analysed by 12 laboratories of which four used the CALUX assay, five the DR-CALUX assay, while the H4IIE-luc, the EROD micro bioassay and the MH1C1 EROD assay were used by one laboratory each. The concentration of TEQs determined by the laboratories ranged between 0.2 and 28.7 pg/g fresh weight (table 1). The TEQ mean for the CALUX bioassay was 17.6 pg/g and the mean for the DR-CALUX bioassay was 17.0 pg/g. Overall, eight of the twelve laboratories had values that were between 60 and 106% of the WHO-TEQ value of 27 pg/g

wet weight. This is considered to be a relatively good agreement. Most of the laboratories were thus able to predict the WHO-TEQ fairly well, however almost all participants reported values below the WHO-TEQ value. The reason for this remains to be clarified, but one explanation could be antagonistic effects of mono- or di-ortho PCBs [6, 7]. It is for instance well known that mono-ortho PCB such as PCB 118 (present in high levels in the cod liver) may act as AhR antagonist in some bioassays [6]. Thus, non ortho and di-ortho PCBs present in the extracts may have exerted antagonistic effects on the bioassay response, leading to lower responses than calculated by the WHO-TEF approach.

No consistent differences in TEQ levels between the different bioassay types could be seen. This is not surprising, since the bioassays used to perform the cod liver analysis all are based on rat and mouse hepatomas. Another observation was the fairly low variability in the bioassay performance within the different laboratories, with RSDs (relative standard deviations) for seven of the laboratories being below 25%. Looking at different bioassays, it can be seen that the RSD for the CALUX bioassay was between 5 and 31% (data from 4 laboratories), while the RSD for the DR-CALUX was between 3 and 44% (data from 5 laboratories).

No specific bioassay type proved to have a lower RSD than any of the other bioassay types. The variability within each laboratory is probably due to the performance of the technical equipment used and of the technicians doing the bioassay analysis.

Table 1. Concentrations of TEQs in the cod liver sample, determined in the different bioassays. Three to four independent assays were performed to determine the TEQ-level.

		Cod liver (pg TEQ/g fresh weight)					
Lab code	Bioassay type	assay 1	assay 2	assay 3	assay 4	Mean	SD
1	CALUX	20.9	16.2	13.4		16.8	3.8
3	DIPS-CALUX[1]	24.5 (6.3)	29.1 (5.0)	27.1 (4.7)		26.9	2.3
15	CALUX	19.7	16.6	10.1		15.5	4.9
8	DIPS- CALUX[1]	11.4 (4.6)	10.5 (3.6)	11.5 (3.9)		11.1	0.6
2	DR-CALUX	26.9	24.6	21.4		24.3	2.8
13	DR-CALUX	28.2	23.8	10.8		20.9	9.0
11	DR-CALUX	20.7	22.2	12.9		18.6	5.0
16	DR-CALUX	1.5	1.1	2.7		1.8	0.8
10	DR-CALUX	19.9	18.5	19.4		19.3	0.7
7	H4IIE-luc	0.1	0.2			0.2	0.1
12	EROD micro bioassay	17.0	23.0	18.0	17.0	18.8	2.9
4	MH1C1 EROD assay	32.0	24.0	30.0		28.7	4.2

[1]In the DIPS-CALUX, the sample clean up is said to allow separation of PCBs from PCDD/Fs. Values given are the total TEQs from the PCDD/F and the PCB fraction. Values within the brackets are the TEQ contribution from the PCB fraction.

Conclusions

Many of the laboratories were able to predict the WHO-TEQ level in the cod liver with a relatively good degree of accuracy. Overall, eight of the twelve laboratories had values that were between 60 and 106% of the WHO-TEQ value of 27 pg/g wet weight. This must be considered to be in good agreement with the chemical analysis with GC/MS. However, in order to reduce the risk for false negatives, the TEQ contribution in the bioassays of partial AhR agonists and

antagonists like mono- and diortho PCBs requires further attention. It should however be noted that non-additive interactions is a limitation in the WHO-TEF approach and not in the bioassay approach, which always reflects the combined effect of all AhR-interacting compounds in a sample.

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