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COMPARISON OF CALUX-TEQs AND LEVELS OF PCDD/F AND PCB IN SFE-EXTRACTS OF HUMAN ADIPOSE TISSUE

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Introduction

Dioxin bioassays have been used as an alternative and complement to conventional high resolution gas chromatography/mass spectrometer (HRGC/MS) analysis of human samples in epidemiological studies[1]. Dioxin bioassays are sensitive and cheaper than chemical analysis, enabling larger numbers of samples to be run. These conditions make dioxin bioassays like the CALUX well suited to conduct large-scale epidemiological studies. However, even though the analysis in itself is time effective, the extraction and clean up is still throughput limiting, and the choise of clean up method may be very crucial for the results obtained[2]. Presence of Ah receptor (AhR) interacting and inhibiting compounds in a sample subject to bioassay analysis will obstruct the interpretation of results and may lead to under- or overestimation of TEQs[3,4]. One way to estimate TEQ content by bioanalytical methods more appropriately, is to separate compounds of interest by chemical fractionation of samples.

In order to improve throughput capacity, new extraction techniques with automation possibilities and decreased time and solvent consumption have been developed for analysis of PCDD/Fs[5,6]. The mostly used extraction techniques like Soxhlet often use large solvent volumes and require long extraction times. The supercritical fluid extraction technique (SFE) has seen an increased interest as an environmental friendly sample preparation technique and it is now well established as a technique with short extraction times and minimal usage of organic solvents for chemical analysis.

The use of the CALUX bioassay[7] in combination with the rapid and automated SFE-LC extraction and fractionation technique to screen large numbers of human samples holds promise for future epidemiological studies. In this study, we have tested the applicability of this combination of techniques on planar and non-planar fractions of human adipose tissue samples. We tested the two fractions separately and together, to test the assumption of additive effects of dioxin-like compounds. In addition, we have compared the CALUX TEQs levels with PCB and PCDD/F measurements, expressed as WHO-TEQs and TEQs (i.e. TEQs calculated using CALUX-specific relative potency values).

Methods and Materials

Five replicates of a human adipose tissue sample were extracted and fractionated by SFE-LC as described by Lindström et al.[6,8], giving one fraction containing planar Ah- receptor (AhR) agonists (eg PCDD/Fs, non-*ortho*-PCBs and PCNs), and one fraction containing non-planar compounds, (eg mono- and di-*ortho*-PCBs, HCB, DDE, chlordanes and PBDEs).

The two fractions of each replicate were screened for dioxin-like activity in the DR-CALUX bioassay[7]. In addition, fractions were put together and tested, for each replicate. Briefly, DR-CALUX cells were seeded in sterile, transparent 96-well plates, in 100 µl/well aliquots. The minimal essential medium (α-MEM, Sigma) used was supplemented with 10% fetal calf serum. Plates were incubated for 24h, allowing cells to reach 90-100% confluence. The medium was removed before addition of exposure medium. Exposure medium was prepared by adding dimethylsulfoxide (DMSO) solutions of samples to culture medium. 100 µl/well of exposure medium were added to the cells, in triplicate wells. The final concentrations tested were 270-340 mg lw/ml medium. DMSO concentrations in wells were 0.8-1%. In each assay, a standard curve of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was tested (0-300 pM). After 24h exposure, cells were washed twice with phosphate-buffered saline (PBS). Cell lysis and enzymatic reaction (~30 min) were performed with the Luclite kit (Perkin Elmer). Cell lysates were transferred to white 96-well plates before the luciferase activity was determined in a plate reader (Victor^2, Wallac, Perkin Elmer). Values reported for the replicates represent a mean of two to three separate analyses.

The non-planar fraction was analysed by GC-LRMS (SIM, EI) for PCBs #118, #114, #105, #156, #157 and #159. The planar fraction, containing PCDD/Fs, non-*ortho*-PCBs and PCNs was analysed for tetra- to octa-PCDD/Fs, by GC-HRMS (SIM, EI)[6].

Results and Discussion

Bioassay and chemical analyses showed comparable results, table 1. For the planar fraction (PCDD/Fs, non-*ortho*- PCBs and PCNs), chemically and biologically derived TEQs were very similar, irrespective of using WHO-TEFs or CALUX-REPs. The ratio ($R_{b/c}$)[9,10] between bioanalytical (b) and chemical (c) results were 0.91 in both cases. The chemical TEQs included PCDDs and PCDFs, but not planar PCBs, therefore the chemical TEQs may be somewhat higher than what is reported here. Hence, the true difference between bioassay and chemical results may be larger, with a lower $R_{b/c}$. A lower $R_{b/c}$ can be explained by non-additive interactions between compounds present in the sample, which are not considered in the chemical calculations. The CALUX assay gave a good estimation of WHO-TEQs in the planar fraction of these samples ($R_{b/c}$ = 0.91). The same correlation was not seen for the non-planar fraction, including mono- and di-*ortho*-PCBs, as calculation using WHO-TEFs gives a 2-fold larger value, compared to CALUX-TEQs ($R_{b/c}$ = 0.41) and CALUX-REP-TEQs. The reason for this difference is the high WHO-TEF values for the mono-*ortho*-PCBs compared to the REP values for these congeners in the DR-CALUX. A very good correlation was seen between the CALUX-TEQs and REP-TEQs ($R_{b/c}$ = 1.28) indicating that the compounds in this fraction had an additive effect, even though several mono- and di-*ortho*-PCBs have been shown to have an antagonistic effect in Ah-receptor based bioassays[11,12]. Earlier studies have shown similar $R_{b/c}$ values for CALUX screening of human tissue samples[13].

The additivity were lost when fractions were put together and analysed, and the bioassay could only detect 53% of chemically derived TEQs. This may be explained by competitive inhibition between AhR agonists and partial agonists[14]. An alternative explanation could be an increased metabolism of non-planar compounds when the fractions are put together. Planar compounds are more potent inducers of metabolic enzymes, eg CYP1A, which might result in lower concentrations of non-planar AhR agonists after 24 hours exposure, compared to separate analysis

of the non-planar fraction. Thus, bioassay analysis of unfractionated and more complex samples may not reveal the total content of dioxin-like compounds and other AhR agonists, due to complex interactions between different groups of compounds. On the other hand, in epidemiological studies, testing of unfractionated samples may be more relevant for estimations of internal exposure.

The SFE-LC extraction and fractionation technique was rapid and used small solvent volumes and yielded well-defined fractions that could be used without subsequent clean-up in chemical and bioassay analysis of dioxin-like compounds[6]. Thus, the SFE-LC extraction and fractionation technique is very suitable to use in combination with dioxin bioassays to screen human adipose tissue samples. Although very low TEQ levels (~10-20 pg TEQ/g lw), the CALUX assay showed good reproducibility.

Table 1. TEQ-levels in planar and non-planar fractions of a human adipose tissue sample (five separately extracted replicates). Fractions were tested separately and together in the CALUX assay. A comparison between bioassay CALUX-TEQs, and chemical REP-TEQs and WHO-TEQ. $R_{b/c}$= ratio bioassay analysis/chemical analysis.

Replicate no	1	2	3	4	5	Mean ± SD	$R_{b/c}$
Planar							
CALUX-TEQ[1]	10.8	7.1	10.2	9.3	6.6	8.8 ± 1.7	
REP-TEQ[2]	8.5	10.3	10.1	10.0	9.5	9.7 ± 0.6	0.91
WHO-TEQ[3]	8.3	10.4	9.9	10.2	9.7	9.7 ± 0.7	0.91
Non-planar							
CALUX-TEQ[1]	7.8	9.6	8.2	13.1	9.2	9.6 ± 1.9	
REP-TEQ[2]	6.3	8.1	7.0	7.5	8.6	7.5 ± 0.8	1.28
WHO-TEQ[3]	19	24	22	24	26	23 ± 2.4	0.42
Planar + non-planar							
CALUX-TEQ[1]	10.2	6.0	11.3	12.9	5.6	9.2 ± 2.9	
REP-TEQ[2]	14.8	18.4	17.1	17.5	18.1	17.2 ± 1.3	0.53

[1] measured with the DR-CALUX bioassay
[2] calculated from chemical analysis and DR-CALUX specific relative potency values
[3] calculated from chemical analysis and WHO-TEFs

Conclusions

The SFE-LC extraction and fractionation technique is very suitable to use in combination with dioxin bioassays to screen human adipose tissue samples for dioxin-like activity, with respect to time effectiveness and reproducibility. A good correlation between CALUX-TEQs and chemically derived REP-TEQs is seen for both the planar fraction and the non-planar. This indicates an additive effect of compounds present, an additivity that is lost when fractions are put together and tested, yielding a more complex exposure. For the planar fraction, CALUX-TEQs and REP-TEQs also correlate well with WHO-TEQs, whereas the WHO-TEQ is 2-fold higher for the non-planar fraction.

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