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Measuring TCDD Equivalents in Environmental Samples with the Micro-EROD Assay: Comparison with HRGC/HRMS Data

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Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F) are ubiquitous environmental contaminants which cause a multitude of effects such as hepatotoxicity, tetratogenesis, immunotoxicity and tumorigenesis (Devito and Birnbaum 1994). The toxicology assessment of mixtures of PCDD and PCDF is based on the 2,3,7,8-TCDD toxicity equivalents (TEQ) which are calculated from the levels of the 17 PCDD/F congeners with 2,3,7,8-chlorine substitution (Safe 1990). At present, gas chromatography combined with mass spectrometry (HRGC/HRMS) is the standard method for quantitating PCDD/F.

When a large number of environmental samples is to be analyzed, however, this techniques is expensive and time consuming. Moreover, only 17 PCDD/F are restricted in the chemical analysis, toxic effects of similar compounds like coplanar PCB congeners, fluorinated, brominated, and mixed PCDD/F are not accounted for. As a consequence, efforts were made to develop biological methods which are more rapid, simple and less expensive than HRGC/HRMS method for the determination of toxic potency. Due to the good correlation between the CYP1A1 induction potency of individual PCDD/F congener and their toxicities, a bioassay has been established measuring the induction of CYP1A1 associated ethoxyresorufin-O-deethylase (EROD) activity, predominantly using rat hepatoma cells H4IIE, for determination of TEQ potency (Tillitt et al. 1991; Safe 1993; Wu et al. 1996). However, this EROD assay is measured in microsomes prepared from homogenized cell samples. Harvesting monolayers, disruption of cells, and preparation of subcellular fractions require precise handling of samples and involve a relatively long working time to produce reproducible results. Because of this, experiments need to be performed in large culture plates and, therefore, a considerable number of cells are required for each experiment. In order to reduce the cost and time in measurement, sensitive micro-assays are developed based on the conventional EROD-assay, for measuring EROD activity in intact cell cultures using multiwell plates (Donato et al. 1993; Kennedy et al. 1995; Hahn et al. 1996). These micro-assays require no

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cell disruption and allow repeated assays with the same cell monolayer. However, a further simplification and additional application of these micro-EROD-assays are still very useful.

In the present study, the micro-EROD assay developed by Donato et al. (1993) was adapted and simplified for determining EROD activity in 96-well plates with rat H4IIE hepatoma cells. Several kinds of environmental samples from China and Germany were analyzed by this improved micro-EROD bioassay followed by a simple "sandwich" column clean-up. In paralleling, TEQ values were determined using the full clean-up procedure and HRGC/HRMS. The reliability of the EROD bioassay was evaluated by comparing the two data sets.

MATERIALS AND METHODS

Environmental samples were fly ash, sediment, chimney soot, and fish liver which were taken from typical sites in China and Germany. The samples were freeze-dried and pulverized. About 1-5g aliquots were extracted in a soxhlet apparatus using toluene for 24 hours. For chemical analysis, the samples were spiked prior to extraction with $^{13}C_{12}$ labeled 2,3,7,8-substituted PCDD/F. Cleanup of samples and quantification of PCDD/F using capillary HRGC/HRMS were done as described by Wu et al. (1997). The MS-measurement was conducted with high resolution at Finnigan MAT 95 (R=10000) for isomer specific measurement.

For clean up of EROD bioassay, a simple "sandwich column" chromatographic purification was applied (Wu et al. 1996). A column was wet-filled from bottom to top with 10g silica, 20g silica (44% concentrated sulfuric acid w/w), and 40g silica (4% water w/w). The column was topped with anhydrous Na_2SO_4. Samples were eluted with 870ml n-hexane and eluate was reduced by evaporation (550mbar,333K) to 2-3ml. The extract was transferred stepwise into a vial and evaporated to dryness under a stream of nitrogen. Samples were redissolved in 500 µl of DMSO:isopropanol (4:1 v/v). Recovery levels for the extraction and clean-up of samples for bioassay were ranged from 90% to 105%.

Rat hepatoma cells H4IIEC3/T (H4IIE) were obtained from E.B. Thompson (National cancer institute, Bethesda, MD, USA). Cells were grown in Dulbecco's Minimum Essential Medium, (supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml strepomycin), at 95% relative humidity, 37 ℃ in air containing 5% CO_2 (Roscher and Wiebel 1992).

The EROD activity in intact cells seed on 96 well plates was determined according to Donato et al. (1993) modified as follows: Cells were seeded at a density of 1×10^4/well in a 96-well plate. After 3 days the medium was replaced

with 100μl medium containing samples extract or TCDD standard. After 72 h the medium was removed and 100μl fresh medium containing 8 μM 7-ethoxyresorufin and 10 μM dicumarol were added. After incubation for 60min at 37℃, the medium was transferred to a fresh 96-well plate and 130μl methanol were added. Resorufin-associated fluorescence was measured in these solutions on a multiwell fluorescence reader (Fa. Fluoroskan II, Fa. Labsystem). The 96-well plate containing the remaining live cell cultures was for a cytotoxicity test using the neutral red assay (Babich. 1986). Thereafter the protein content per well was assayed using bicinchoninic acid according to Smith et al. (Smith et al. 1985). Standard deviations between triplicates remained less than 10%.

The biological TEQ values were determined according to Hanberg et al. (1991) by comparing the induction of EROD activity caused by environmental sample extracts with that caused by authentic TCDD standards. The average deviation between duplicate measurements was ± 25%.

RESULTS AND DISCUSSION

For determination of EROD-TEQ in unknown samples, a standard curve was used which is based on the induction value of 2,3,7,8-TCDD standards covering 0 to 100% of induction of a control with intact H4IIE cells in 96-well plates. Figure 1 shows the resulting standard curve for 2,3,7,8-TCDD with micro-EROD assay in present study. The EC50 is 0.9 pg 2,3,7,8-TCDD /ml, and the standard deviation is less than 5%. The detection limit in intact H4IIE cells is 0.4 pg 2,3,7,8-TCDD/ml, which equals an absolute amount of 40 fg 2,3,7,8-TCDD /well in 96-well plates. It was reported by Wu et al. (1996) who used conventional EROD assay with H4IIE hepatocyte microsomes that the detection limit was 2.5 pg 2,3,7,8-TCDD /well in 24-well plates. Obviously, the detection limit in this micro-EROD-assay is a factor of 60 lower than that obtained in conventional EROD-assay and comparable to the CALUX assay with a detection limit of 32 fg/well (Murk et al. 1997). Thus, this micro-EROD-assay is much more sensitive. Moreover, like another EROD bioassay performed in PLHC-1 fish hepatoma cells using 48-well plates (Hahn et

al. 1996), this micro-EROD assay procedure avoids collection of cells and preparation of in vitro incubation mixtures requiring an exogenous source of NADPH, as practiced by others (Kennedy et al 1993; Wu et al. 1996), thereby, producing considerable time and cost savings. It is also preferable to other 96-well plated-based procedures involving the use of frozen/thawed cells (Kennedy et al. 1993; 1995), since the intact cells allow additional test to be conducted.

The clean up using a sandwich column was tested for its applicability using 16 samples derived from a variety of matrix, such as fly ash, chimney soot, electrode

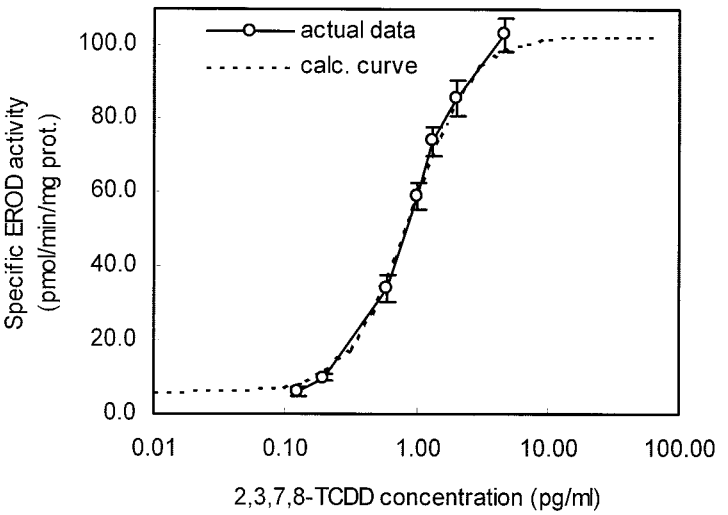

Figure 1. Dose-response relationship for 2,3,7,8-TCDD with micro-EROD-assay in intact rat hepatocytes with 96-well cell culture plates. Cells were exposed to 2,3,7,8-TCDD in DMSO:isopropanol (0.5% v/v, in medium) for 72h. Error bars represent the standard deviations, n=19.

sludge, wood, sediment, and fish liver. Under the present condition that samples applied to column did not exceed 10g, the clean up could remove all potentially cytotoxic components from the samples. No cytotoxicity could be detected in the final eluates (data not provided). Also, the isolated material interfered with neither the induction nor the assay of EROD activity.

In order to obtain reliable bioassay results (micro-EROD TEQ), sample extracts were usually diluted to different concentrations until the response could be found in the effective region of standard curve. Results derived from the standard curve should be related to original sample concentration by back calculation using the proper dilution and volume factors. For the chemical analysis, the PCDD/F TEQ was calculated using the TCDD-equivalent factors (TEF) from 17 PCDD/F congeners with 2,3,7,8-chlorine substitution (Safe 1990). Figure 2 shows the biological TEQ values in several environmental matrix derived from micro-EROD-assay, and their chemical TEQ values derived from HRGC/HRMS analysis.

For all environmental samples the biological TEQs are higher than the values from chemical analysis by a factor of 1.1-3.4. This is due to the fact that bioassay measures the response to all potential toxic compounds that are able to bind to Ah receptor and thereby induce CYP1A1, such as polychlorinated biphenyls (PCB), polyhalogenated azo and azoxy compounds, biphenyl ethers, naphthalenes, sulfur-analogue dioxins, and alkylated, brominated dibenzodioxins.

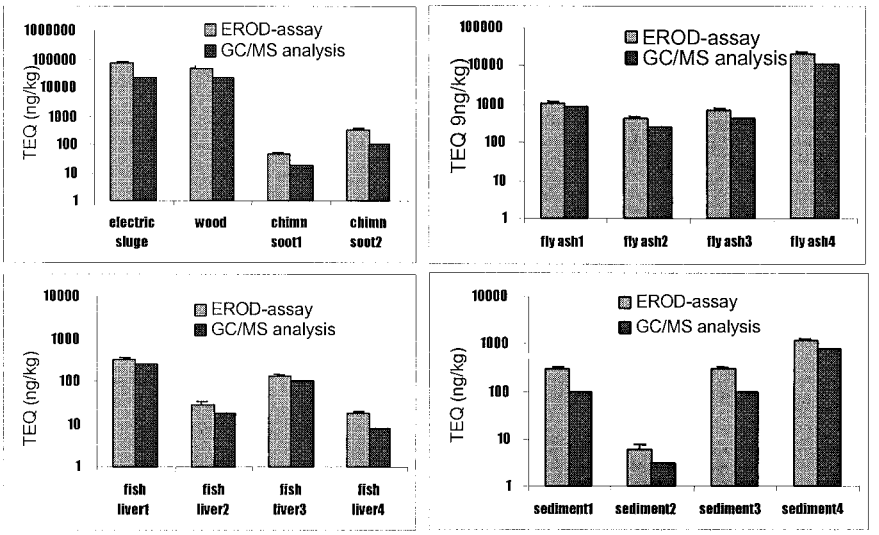

Figure 2. Comparative results of micro-EROD-assay and HRGC/HRMS analysis in several environmental samples. Error bars represent the standards deviation with n=4

The comparative results give a further confirmation that the single cleanup step is sufficient for eliminating a host of substances that might interfere with the bioassay, but does not remove compounds that are frequently associated with PCDD/F.

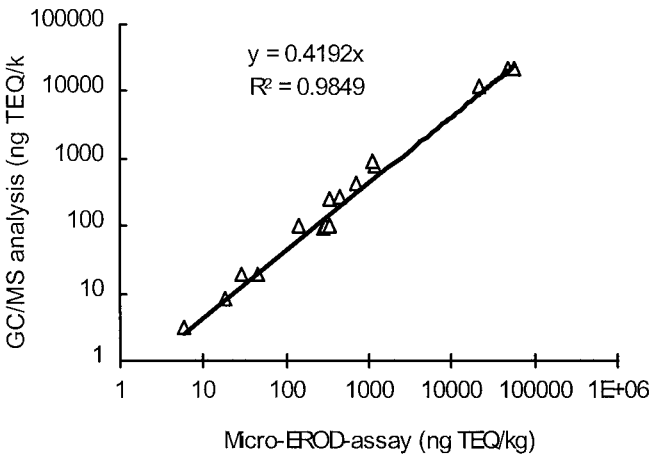

Figure 3. Correlation between bioassay TEQs and chemical TEQs in several environmental samples.

Figure 3 presents the correlation between bioassay TEQs and chemical TEQs in these environmental samples. The bioassay TEQs correlated very well with chemical TEQs (r^2=0.9849, $p<0.05$). The highest TEQ value was observed in one electrode sludge sample amounting to 21650 ng/kg (HRGC/HRMS) and 56332 ng/kg as determined by the bioassay. The least contaminated sediment sample contained 3.1 ng/kg (HRGC/HRMS) and 6.0 ng/kg (bioassay). It is obvious this micro-EROD-assay has a very wide range for TCDD TEQ measurement. Because the bioassay consistently yields higher TEQ values than chemical analysis, it is highly unlikely to produce false-negative results. Thereby, the bioassay can be used as a rapid screening tool for large numbers of environmental samples with various matrix.

Our results show that the micro-EROD-assay is a rapid and sensitive method to determine the AhR-related toxic potency in intact H4IIE hepatocyte cells in 96-well plates. The good correlation between the results from micro-EROD and HRGC/HRMS confirms that the micro-EROD-assay with its special cleanup is sufficiently reliable for the determination of TEQ in environmental samples. As no time consuming steps are required in the sample preparation and measurement, it is suitable as a tool for rapid screening of large quantities of environmental samples in order to selected critical samples for detailed investigation by HRGC/HRMS analysis.

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REFERENCES

Babich H, Borenfreund E (1986) Structure-activity relationship (SAR) models established in vitro with the neutral red cytotoxicity assay. Toxicol in Vitro 00: 1-7

Devito MJ, Birnbaum LS (1994) Toxicology of dioxins and related chemicals, in dioxins and health (A. Schechter, Ed.). Plenum, New York, p139-162

Donato MT, Gomez-Lechon MJ, Castlell JV (1993) A microassay for measuring cytochrome P4501A1 and P450 1B1 activities in intact human and rat hepatocytes cultured on 96-well plates. Anal Biochem 213: 29-33

Hahn ME, Woodward BL, Stegeman JJ, Kennedy SW (1996) Rapid assessment of induce cytochrome P4501A protein and catalytic activity in fish hepatoma cells grown in multiwell plates: response to TCDD, TCDF, and two planar PCBs. Environ Toxicol Chem 15: 582-591

Hanberg A, Strahlberg M, Georgellis A, de Wit C, Ahlborg UG (1991) Swedish dioxin survey: envalution of the H4IIE bioassay for screening

environmental samples for dioxin-like enzyme induction. Pharmacol Toxicol 69: 442-449

Kennedy SW, Lorenzen A, James CA, Collins BT (1993) Ethoxyresorufin-O-deethylase and porphyrin analysis in chicken embroy hepatocyte cultures with a fluoresecence multiwell reader. Anal Biochem 211: 102-112

Kennedy SW, Jones SP, Bastien LJ (1995) Efficient analysis of cytochrome P4501A catalytic activity, porphyrins, and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. Anal Biochem 226: 362-370

Murk AJ, Leonards PEG, Bulder AS, Jonas AS, Rozemeijer MJC, Denison MS, Koeman JH, Brouwer A (1997) The CALUX (chemical-activated luciferase expression) assay adapted and validated for measuring TCDD equivalents in blood plasma. Environ Toxicol Chem 16: 1583-1589

Roscher E, Wiebel FJ (1992) Genotoxicity of 1,3- and 1,6-dinitropyrene: induction of micronuclei in a panel of mammalian test cell lines. Mutat Res 278: 11-17

Safe S (1990) Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit Rev Toxicol 21: 51-88

Safe S (1993) Toxicology, structure-function relationships, human and environmental health impacts of polychlorinated biphenyls (PCBs): progress and problems. Environ Health Perspect 101: 317-322

Smith PK, Krohn RJ, Hermanson GT, Malia AK, Gartner FH, Prouanzano MD (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76-85

Tillitt DE, Ankley GT, Giesy JP (1991) Characterisation of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. Environ Sci Technol 25: 87-92

Wu WZ, Schwirzer SMG, Schramm KW, Wiebel FJ, Xu Y, Zhang YY, Yediler A Kettrup A (1996) Induction of ethoxyresorufin O-deethylase (EROD) as indicator of potentially harmful effects for dioxin-like compounds in environmental samples of Ya-Er Lake area, China. Fresenius Environ Bull 5: 374-379

Wu WZ, Schramm K-W, Henkelmann B, Xu Y, Yediler A, Kettrup A (1997) PCDD/Fs, PCBs, HCHs and HCB in sediments and soil of Ya-Er Lake area in China: results on residual levels and correlation to the organic carbon and the particle size. Chemosphere 34: 191-202