A Bioassay (EROD-Assay) for Measuring TCDD Equivalents (TEQ) in Environmental Samples: Comparison to a Microassay and to Chemical Analysis.

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Introduction

Polyhalogenated aromatic hydrocarbons (HAH), such as polychlorinated dibenzo-*p*-dioxins (PCDD), dibenzofurans (PCDF) and biphenyls (PCB) are industrial compounds or by-products that are widespread in the environment. In addition, they are released from incomplete combustion processes (e.g. in fly ash). Because of their highly liphophilic character and their resistance to metabolism and to chemical degradation, they accumulate in the food chain and may elicit toxic effects. In *in vivo* studies, effects include dermal toxicity, hepatotoxicity, teratogenesis, immunotoxicity and tumor promotion [1].

The most toxic compound is 2,3,7,8-TCDD. Based on *in vivo* and *in vitro* studies, relative toxicities for other substances have been determined relative to TCDD [2]. The total toxic equivalent (TEQ) is defined as the sum of the products of the concentrations of individual isomers and congeners of PCB, PCDD and PCDF, and their toxic equivalency factors (TEF) [3]. Up to now gas chromatography combined with mass spectrometry has been the standard method for quantitating HAH. When a large number of environmental samples is to be analyzed, however, these techniques are too expensive and time consuming. Moreover, routine chemical analysis considers only 17 chlorinated PCDD/F. Similar toxic compounds like fluorinated, brominated and mixed halogenated PCDD/F, as well as other polyhalogenated compounds such as, azo- and azoxy-compounds, biphenylethers, naphtalines, sulphur-analogue dioxins and alkylated dibenzofurans are not taken into account. Because of these deficits a quick and cost effective alternative was developed. The aim was to obtain TCDD-TEQ that integrate the biological effects of complex mixtures of PCB, PCDD, PCDF and other HAH [2]. Considering these facts a bioassay has been developed, using rat hepatoma H4IIE cells. This system is sensitive to cytochrome P4501A (CYP1A) induction, an Ah-receptor-mediated re-

sponse that is associated with many of the biochemical and toxic effects of PCB, PCDD and PCDF [4].

The toxic potencies of HAH congeners in rats correlate quite well with their abilities to induce CYP1A catalytic activity in rat hepatoma H4IIE cells [5].

In order to reduce the cost and time in screening a great number of environmental samples, we attempted to implement a sensitive assay developed by Donato et al.[6] for measuring cytochrome P4501A activities in intact rat hepatocytes. Since this assay does not destroy the hepatocytes, it provides the possibility of either performing repeated assays in the same monolayer, or using the intact cells for investigations of cytotoxic effects.

Several matrices were examined with both EROD- and Micro-EROD-assays in order to determine whether sufficient correlation exists between the two screening methods and thus to establish the microassay as a possible alternative.

Methods

Test materials: The fly ash and the tissue filter dust samples were collected at a municipal waste incineration plant. The sludge samples were collected at a sewage sludge plant in northern Bavaria, FRG.

Sample preparation and extraction: 5-10 g aliquots were Soxhlet extracted using toluene for 24 hours. For chemical analysis, the samples were spiked prior to extraction (isotope dilution method) with a standard containing seventeen $^{13}C_{12}$-labelled PCDD/F.

Determination of PCDD/F and PCB TEQ by chemical analysis: Cleanup and quantification were done as described elsewhere [7] using capillary HRGC/HRMS. The MS-measurement was conducted under high resolution using a Finnigan MAT 95 (R=10000) instrument for isomer specific measurement.

Cleanup procedure for the EROD- as well as for the Micro-EROD-assay: Concentrated crude extract was applied to a column filled with n-hexane from bottom to top with 10 g silica, 20 g silica (44 % conc. sulfuric acid w/w), 40 g silica (4% water w/w). The column was topped with Na_2SO_4. Active silica mesh 63-200 µm was used. Samples were eluted with 870 ml n-hexane and the eluate was reduced by evaporation (550 mbar, 343 K) to 2-3 ml. The extract was transferred stepwise into a vial and evaporated to dryness under a stream of nitrogen. Then the samples were redissolved in 500 µl of DMSO:isopropanol (4:1v/v).

Cell culture and EROD/Micro-EROD-assay: H4IIE/T cells were routinely grown in Dulbecco's Minimum Eagles Medium with 5% calf serum [8]. For the EROD-assay they were seeded at a density of 1 x 10^5/50mm culture dish. After attaining a density of about 70 % cells were exposed to the environmental samples (5µl per 1 ml medium) or standards of 2,3,7,8-TCDD. After 72 h of exposure cells were harvested and CYP1A activity was determined by the method of Pohl and Fouts [9]. For the Micro-EROD-assay cells were seeded at a density of

1 x 10^4/well in a 96-well plate. CYP1A activity was measured after a 72 h exposure using a method of Donato et al.[6].

Results and Discussion

Table 1, 2 and 3 compare the TEQs derived from EROD-assay and from chemical analysis of ten samples to the values obtained from Micro-EROD-assay. The biological TEQs from all samples were significantly higher than those obtained by chemical analysis. This difference is due to the fact that the result of the bioassay includes the biological response to compounds like polyhalogenated azo- and azoxy-compounds, biphenylethers, naphthalines, sulphur-analogue dioxins and alkylated dibenzofuranes which also bind to the Ah-receptor and thereby induce cytochrome P4501A, while those substances are not detected routinely by chemical analysis. Moreover, TEF values for such compounds have been unknown up to now.

Table 1: TEQs of one fire residue and two fly ash samples : comparison of EROD-assay results with those of Micro-EROD-assay and chemical analysis

	EROD-assay	Micro-EROD-assay	chemical analysis
fire residue	981 ± 5 [a]	1067 ± 47 [a]	880 [c]
fly ash 1	630 ± 24 [a]	450 ± 27 [a]	264 [b]
fly ash 2	611 ± 15 [a]	705 ± 35 [a]	416 [c]

[a] mean ± standard deviation of triplicate determinations, [b] TEQ (sum of PCDD/F and PCB) PCDD/F according to NATO/CCMS PCB according to WHO, [c] TEQ (PCDD/F) according to NATO/CCMS

The validity of the Micro-EROD-assay was examined by comparing the results obtained in intact cultured hepatocytes with the activitiy determined in the conventional EROD-assay. By measuring monooxygenase activities directly in intact cells, the loss of biological activity is avoided and the assay time is reduced drastically. Moreover, in contrast to the assay using intact cells, addition of cofactors like NADPH-regenerating systems is not needed since hepatocytes generate their own NADPH.

A good correlation between the two assays was found for the fire residue, fly ash 1 and fly ash 2 (table 1) as well as for tissue filter dust 1 (table 2). For the samples of tissue filter dust 2 and tissue filter dust 3 (table 2) there is a small deviation between the TEQs derived from Micro-EROD-assay in comparison to those obtained from EROD-assay. Nevertheless, the correspondence to chemical analysis is quite good.

Table 2: TEQs of three tissue filter dust samples: comparison of EROD-assay results with those of Micro-EROD-assay and chemical analysis

	EROD-assay	Micro-EROD-assay	chemical analysis
tissue filter dust 1	1695 ± 7 [a]	1460 ± 26 [a]	1168 [b]
tissue filter dust 2	1233 ± 14 [a]	735 ± 45 [a]	638 [b]
tissue filter dust 3	2617 ± 39 [a]	2015 ± 163 [a]	1645 [b]

[a] mean ± standard deviation of triplicate determinations, [b] TEQ (sum of PCDD/F and PCB) PCDD/F according to NATO/CCMS, PCB according to WHO

The TEQ values obtained from EROD- and Micro-EROD-assay correspond well for the sludge samples (table 3). There also could be determined a good correlation to chemical analysis.

Table 3: TEQs of four sludge samples: comparison of EROD-assay results with those of Micro-EROD-assay and chemical analysis

	EROD-assay	Micro-EROD-assay	chemical analysis
sludge 1	155 ± 6 [a]	147 ± 9 [a]	59 [b]
sludge 2	139 ± 5 [a]	157 ± 35 [a]	58 [b]
sludge 3	157 ± 3 [a]	189 ± 8 [a]	62 [b]
sludge 4	160 ± 3 [a]	144 ± 16 [a]	54 [b]

mean ± standard deviation of triplicate determinations, [b] TEQ (sum of PCDD/F and PCB) PCDD/F according to NATO/CCMS, PCB according to WHO

The above results show that the Micro-EROD-assay is a sensitive alternative for rapid screening of the toxic potential of large numbers of environmental samples. It was not the aim of these investigations to replace chemical analysis but to find a useful combination of the two determination methods. Only samples surpassing the relevant threshold for the corresponding matrix are subjected to the more costly and time consuming chemical analysis. The combination of these two methods provides an efficient means of characterization and risk assessment.

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