Induction Equivalency Factors for Halogenated Aromatic Hydrocarbons and Polynuclear Aromatic Hydrocarbons Using the H4IIE Rat Hepatoma Bioassay

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1. Introduction

The H4IIE rat hepatoma bioassay has been extensively used and validated for risk assessment of environmental samples containing halogenated aromatic hydrocarbons (HAHs)[1,2,3]. The bioassay quantitates the induction potency of environmental extracts by measuring CYP1A1-mediated induction of ethoxyresorufin-O-deethylase (EROD) activity. CYP1A1 induction is mediated via the aryl hydrocarbon receptor (AhR) signaling pathway. HAHs bind to the same receptor and exhibit similar dose-response induction potencies in both in vitro induction assays and in vivo bioassays. Therefore, a toxic equivalency (TEQ) approach has been developed to describe the potency of complex mixtures of HAHs. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent inducer in H4IIE cells and is used as the standard to develop toxic equivalency factors (TEFs) for the individual HAHs[4,5].

Some environmental samples, such as marine sediments and invertebrate species, accumulate both HAHs and PAHs. Many of the toxic and genotoxic effects of PAHs require metabolic activation, however some PAHs are AhR agonists and potent inducers of EROD activity. Previous research in this laboratory has indicated that PAHs can dominate induction equivalents estimations for oyster samples containing PAHs and HAHs[6]. Therefore, to accurately calculate induction equivalents (EQs) of environmental samples, equivalency factors (EFs) for both individual HAHs and PAHs must be considered as indicated in the following equation:

$$EQs = \Sigma[HAH]_1EF_1 + \Sigma[HAH]_2EF_2 \ldots + \Sigma[PAH]_1EF_1 + \Sigma[PAH]_2EF_2 \ldots$$

Recently, Kennedy and Jones[7] have described a method to determine both EROD activity and protein determination with a multi plate reader. This method allows for rapid determination of EFs where up to seven dilutions of an extract can be read in triplicate on a single plate. Differences between the newer 48-well plate method and the older method where cells were harvested from 6 well plates for EROD analysis[8] can result in different EFs for PAHs and HAHs.

This study is reports induction EFs for seven PAHs relative to TCDD, the most potent inducer in H4IIE cells. Furthermore, the differences between EFs for PAHs and the PCB congeners 77, 126, and 169 derived from the 6 well and the 48 well H4IIE bioassays are compared.

2. Materials and Methods

H4IIE Bioassay 6-Well Method. Rat hepatoma H4IIE cells were grown as a continuous cell line in α-minimum essential media supplemented with 2.2 g/L sodium bicarbonate, 5% fetal bovine serum, and 10 ml/L antibiotic/antimycotic solution. Stock culture cells were grown in 150 cm^2 plates at 37°C in a humidified air/carbon dioxide (95/5%) atmosphere. After confluency, cells from one plate were trypsinized and seeded in six 6-well plates in 2 ml media/well. After 24 hr, cells were dosed with 2 µl PAH or HAH standard so that the final concentration of DMSO was 0.1%. TCDD and PCB 126 standard curves ranged from 10^{-13} M to 10^{-7} M while PAH, PCB 77, and PCB 169 curves ranged from 10^{-5} M to 10^{-10} M. After 24 hr incubation, media was removed, cells washed with 2 ml Hanks' solution and harvested by scraping into 4 ml Tris-sucrose buffer (Tris base 6.05 g/L, sucrose 59.5 g/L, pH 8.0). The cellular pellet was isolated after centrifuging and resuspended in 200 µl buffer. Fifty µl of the cell suspension was used for both EROD assays and Bradford protein determinations[9]. EROD assays were conducted as described by Pohl and Fouts[8] with modification in 1.15 ml incubation mixtures consisting of 0.1 M HEPES, pH 8.0, 0.1 mg NADPH, 0.1 mg NADH, 1.5 mg BSA, 0.7 mg $MgSO_4$, 50 µl of cells. Fluorescent metabolites were determined using a spectrofluorometer at 550 nm/585 nm excitation/emission wavelengths.

H4IIE Bioassay 6-Well Method. Cells were maintained as described above and seeded into 48 well plates at a density of 80,000 cells per well in 0.5 ml media. After 24 hr, plates were dosed with 1 µl per well of HAH or PAH solution. A complete standard curve and a TCDD positive control could be run in triplicate on a plate. The EROD assay and protein determinations were as described[7] on a CytoFluor 2350 plate reader. Plates were read at 530 nm/590 nm for resorufin production and 400 nm/460 nm for fluorescamine protein determination.

EC_{50} Calculation. In the 6-well H4IIE bioassay a Gaussian curve fit was used for TCDD curves and all the PAHs except benzanthracene and chrysene which never reached induction maxima before solubility limitations. For these compounds a logistic curve fit was used. SigmaPlot (Jandel Scientific) was used for these calculations and the equations have been described previously[10]. With the 48-well plate method a Gaussian fit was used for all the PAHs, however a logistic fit was used for TCDD.

3. Results

Figure 1. Illustrates dose-response curves for TCDD, the most, and least potent PAHs in the 6-well H4IIE bioassay. While TCDD and the PAHs reach a similar maxima, chrysene (as well as benzanthracene) did not display a Gaussian distribution. Figure 2 shows the same three compounds using the 48 well plate assay. Using this method TCDD does not exhibit a symmetric Gaussian distribution so a logistic fit was used. However, all of the PAHs maximally induced EROD activity and displayed Gaussian distribution.

EC_{50}s and EFs relative to TCDD for induction of EROD activity are given in Table 1 for both H4IIE bioassay methods. The order of EROD induction potency using the 6 well plates was BkF>BbF>DBA>IdP>BaP>Chr>BA. A different order was observed with the 48 well plate method: BkF>DBA~IdP>BbF>BaP>Chr>BA.

Table 1. Selected PAH Equivalency Factors

	6-Well Plate		48-Well Plate	
	EC_{50} (M)	Induction EF	EC_{50} (M)	Induction EF
BkF	$2x10^{-8}$	0.00478	$4x10^{-10}$	0.0437
BbF	$4x10^{-8}$	0.00253	$4x10^{-9}$	0.0033
DBA	$5x10^{-8}$	0.00203	$1x10^{-9}$	0.0126
IdP	$1x10^{-7}$	0.00110	$1x10^{-9}$	0.00991
BaP	$3x10^{-7}$	0.000354	$3x10^{-8}$	0.000447
Chr	[a]$5x10^{-7}$	0.00020	$7x10^{-8}$	0.000195
BA	[a]$4x10^{-6}$	0.000025	$8x10^{-8}$	0.000180
TCDD	$1.1x10^{-10}$	1	[a]$1.4x10^{-11}$	1

[a]Derived using a logistic function

In the 6 well plates the highest doses of PCB congeners did not cause the characteristic depression of EROD activity, thus a logistic function was used to fit the data, in contrast PCB curves using 48 well plates were Gaussian in distribution. The EFs for PCBs 126 and 169 were within 2 fold for the two methods, however the EF for PCB 77 was 43 times higher using the 48 well plate method.

4. Discussion

EC_{50}s and EF values for HAHs and PAHs from induction of EROD activity were determined in rat hepatoma H4IIE cells. Using the 48 well plate method, higher EFs for several of the PAHs and PCB 77 were observed compared to those determined using the 6 well plate method. The reasons for the differences between the two methods is not understood, however it could be related to differential metabolism of the compounds. The results of this study indicate that determination of induction EQs for extracts containing PAH and HAH mixtures should utilize EFs derived from induction of EROD activity in rat hepatoma H4IIE using the same assay system. Furthermore, because both PAHs and HAHs are potent inducers of EROD activity in this bioassay, the contribution of both classes of inducers should be considered when screening environmental extracts.

5. References

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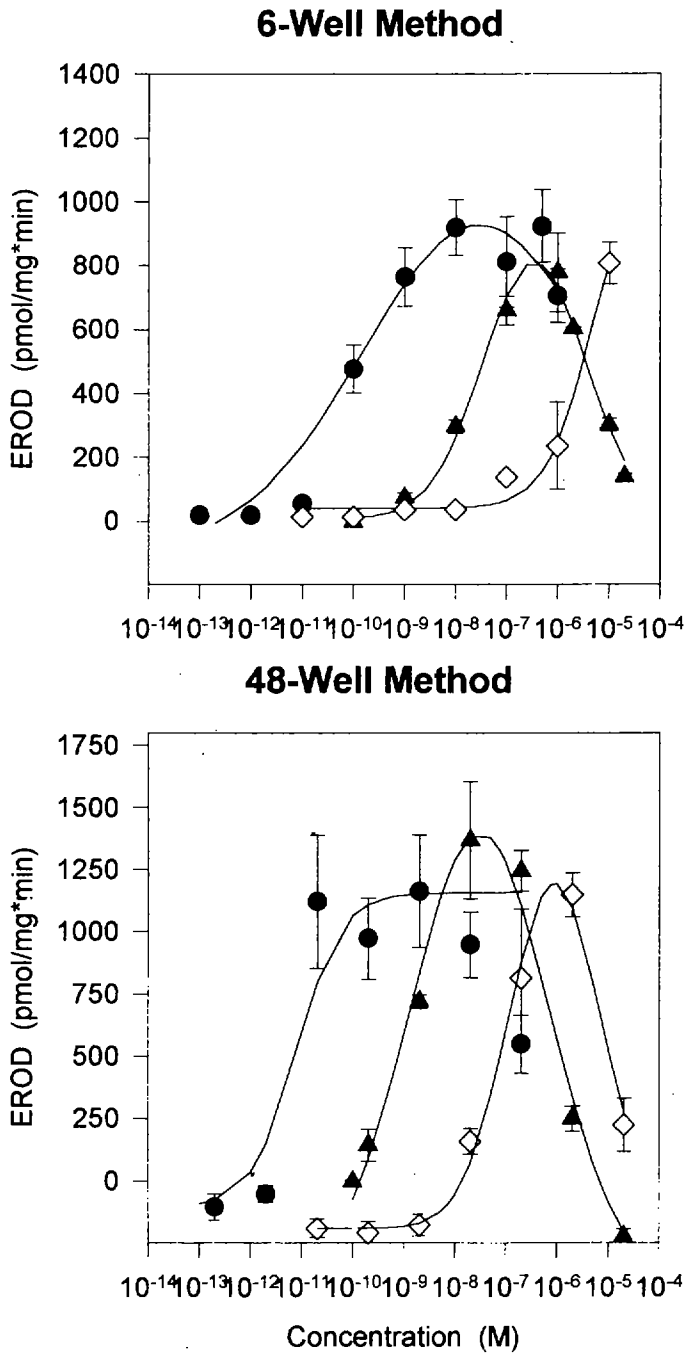

Figure 1. EROD Induction Curves for TCDD, BkF, and BA in the Two Methods

● TCDD ▲ BkF ◇ BA