Novel Quantitative Assessment for the Toxic Effect of Polycyclic Aromatic Hydrocarbon-Like Compounds in a Water Environment Using the Ethoxyresorufin *O*-Deethylase Microbioassay

Seung Min Oh,[a] Byung Woo Ham,[a] Jeoung Han Kim,[b] and Kyu Hyuck Chung*,[a]

[a]*College of Pharmacy, Sungkyunkwan University, 300, Chunchun-dong, Jangan-Ku, Suwon, Kyunggi-do 440–746, Korea and* [b]*School of Agricultural Biotechnology, Seoul National University, 103 Seodundong, Suwon 441–744, Korea*
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The response of environmental pollutants can be detected bioanalytically focusing on the source and matrices of concern. Cell culture bioassays are rapid and inexpensive and thus have great potential for routine monitoring of aquatic resources. Such novel *in vitro* assays are a new tool to investigate lipophilic and low volatile compounds and are a powerful complement to instrumental analysis. The ethoxyresorufin *O*-deethylase (EROD) microbioassay was conducted to determine cytochrome P4501A (CYP1A) activity in environmental samples, and the calculated 3-methylchloranthrene (3-MC) equivalent concentration (MEQ) was introduced as a new quantitative water quality parameter. The chemical MEQ was calculated by multiplying induction equivalency factor (IEF) to GC-MS analysis data. And biological (bio-) MEQ was calculated by comparing the concentration response curve of the sample with those of the 3-MC calibration curve. Therefore, chemical MEQ is an estimation of the toxic effects of polycyclic aromatic hydrocarbons (PAHs) and bio-MEQ is the total toxic effects of various CYP1A-inducing chemicals in water samples. In this study, bio-MEQ values of water samples were higher than chemical MEQ values and total PAH concentrations, indicating that there must be other compounds in the water sample effective as inducers of EROD and that the biological activities of mixture compounds are mainly due to additive effects. There was a good correlation between bio-MEQ and total PAH concentration. The difference between bio-MEQ and total PAH concentration was high in downstream areas, *i.e.*, polluted sites. Bio-MEQ calculated based on the enzyme-inducing effect of water samples could give information about the biological potency of water samples caused by PAH-like compounds.

Key words —— *in vitro* cell culture system, toxicity evaluation, cytochrome P4501A

*To whom correspondence should be addressed: College of Pharmacy, Sungkyunkwan University, 300, Chunchun-dong, Jangan-Ku, Suwon, Kyunggi-do 440–746, Korea. Tel.: +82-31-290-7714; Fax: +82-31-292-8800; E-mail: khchung@skku.ac.kr

INTRODUCTION

Monitoring the levels of contaminants and their geographic and temporal variability is important for assessing the health of the aquatic environment. Chemical analyses are sensitive and specific, but can be expensive and provide little information on the actual or potential biological activity of the contaminants.[1] Biological assays can be used to indicate the presence and potential effects of contaminants in aquatic animals and therefore have potential for routine monitoring of the aquatic environment.[2]

Among aquatic pollutants, polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) comprise a major group.[3,4] The toxicologic relevance of many PAHs lies in their mutagenic and carcinogenic potency, which has been demonstrated in *in vivo* rodent bioassays and *in vitro* short-term bioassays. Because of their mutagenic/carcinogenic character, PAH levels and their effects are regularly monitored using different approaches. A toxic equivalency approach based on an understanding of this mechanism provides an integrated measure of the biological potency or activity of PAH mixtures. Similarly, *in vitro* biomarker responses measured in cell culture bioassays can be used to assess the concentration of toxicity equivalents in extracts of environmental matrices.

The H4IIE rat hepatoma cell line has been used for the evaluation of the relative induction of PCDDs, PCDFs, PCBs and PAHs, and a good correlation between the potency of a number of agonists in the ethoxyresorufin *O*-deethylase (EROD) assay and their relative *in vivo* toxicity in rats has been obtained.[5] A number of attempts has been made to quantify the relative potency of PAHs by establishing toxic equivalency factors (TEFs). Recently, a WHO and The International Programme on Chemi-

cal Safety (IPCS) group of consultants proposed TEFs for metabolizing enzymes, effects on body and organ weights, immunotoxicity, *etc*. For screening purposes, it is generally desirable to use the assay most sensitive for the compound(s) of interest. Ecotoxicologic studies may also focus on ecologic and toxicologic effects observed in the field in retrospective studies in which a causative correlation between effects and chemical residue analysis is often difficult to establish. Commonly, ecologic investigations alone do not have sufficient resolving power to identify the causative agents. Similarly, chemical analysis of pollutants in ecosystems alone cannot provide evidence for toxicologic consequences in biota. Only an integrated approach considering environmental chemical, toxicologic, and ecologic concepts may be suitable for understanding ecotoxicologic effects in contaminated ecosystems.[6)]

In this study, the applicability of an *in vitro* cell system for the toxicity evaluation of PAH-like toxicity is discussed. As an integrated approach for the chemical and toxicologic evaluation, we compared the cytochrome P4501A (CYP1A)-inducing potencies using the toxicity equivalent of 3-methylchloranthrene (3-MC) and determined the chemical composition of carcinogenic PAHs using GC-MS.

MATERIALS AND METHODS

Chemicals —— Standard PAHs were purchased from Sigma Chemical Co. (MO, U.S.A.) Chemicals for the EROD assay, dicumarol and ethoxyresorufin, were purchased from Sigma Chemical Co. PAHs were prepared in 100% dimethyl sulfoxide (DMSO) at a concentration of 10 mM. All chemical solutions were stored at –20°C. Dulbecco's modified Eagle's Medium (DMEM) was purchased from GIBCO BRL. Fetal bovine serum (FBS) was purchased from Hyclone.

Sampling and Pretreatment —— In May, July, September, and November 2000, river water samples were collected from upstream points and downstream points of the Mankyung River in Chunju city and the Gab stream in Daejun city. Fifty liters of river water were adsorbed on an Amberlite XAD-2 resin column. Pollutants adsorbed to the Amberlite XAD-2 resin were extracted by elution with methanol. Ten liters-equivalent extracts were evaporated under a gentle nitrogen flow until only a very small drop remained. To each sample, 100 μl of DMSO was added, and the remaining methanol (MeOH) (100 ml/μl DMSO, final concentration) was completely evaporated.

GC/MS Analysis —— Sixteen PAHs, *i.e.*, naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a*,*h*]anthracene, benzo[*g*,*h*,*i*]perylene, and indeno(1,2,3-*cd*)pyrene, selected by the US Environmental Protection Agency as "Consent Decree" priority pollutants[7)] were analyzed by GC/MS. The mass spectrometer was operated in the selected ion monitoring (SIM) mode. A 30 m 5-MS column with 0.32 mm inner diameter and 0.25 μm film thickness (J&W Scientific Products, Koln, Germany) was used with helium as the carrier gas purity 99.999%). Spiltless injection (1 μl of sample) was performed at 300°C. The temperature of the GC/MS transfer line was 280°C; the oven temperature program was as follows: 65°C for 1 min, 25°C/min to 140°C for 10 min, and 10°C/min to 290°C for 9 min.

Cell Lines —— In this study, H4IIE (rat hepatoma) and RTH-149 (rainbow trout hepatoma) cell lines were used. H4IIE and RTH-149 cell lines were received from the American Type Culture Collection (ATCC). H4IIE cells were grown in MEM supplemented with 5% FBS, penicillin (10000 units/ml), and streptomycin (10000 μg/ml) at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity. RTH-149 cells were grown in MEM supplemented with 10% FBS, penicillin (10000 units/ml), and streptomycin (10000 μg/ml) at 22°C in an atmosphere of 5% CO_2/95% air under saturating humidity.

EROD Microbioassay —— EROD activity was measured using 96-well plates, mostly based on the method described by Drenth *et al.*[8)] with some modifications. Fish cells (RTH-149) and mammalian cells (H4IIE) were seeded in 96-well plates at 3×10^4 cells/well. After 48-hr incubation at 22°C (37°C) when the cell layer was about 80% confluent, 100 μl of DMEM containing the chemical in the maximal 0.1% DMSO was added to each well. After an additional 72-hr (48-hr) incubation the wells were washed twice with phosphate buffured saline (PBS), and dicumarol 5 μM and ethoxyresorufin 4 μM were added. The plates were then incubated again for 2-hr at 22°C (30 min at 37°C). The kinetic conversion of ethoxyresorufin to resorufin was used to estimate the CYP1A1-dependent activity. The resorufin production was measured in a fluorometer

(Perkin-Elmer, LS 50B) with an excitation at 530 nm and emission at 588 nm. The amount of resorufin formed was calculated from a calibration curve for resorufin. After EROD activities were determined, the cells were lysed with 100 μl of 0.1 M EDTA (pH 12.4) and the protein content of the wells was measured using the method of Lowry *et al.*[9] with bovine serum albumin (BSA) as the standard.

Toxicity Equivalent Concentration —— 3-MC (Aldrich), a common EROD inducer, was used as a standard for the estimation of EROD activity equivalency in H4IIE cells. The concentration-response curves of EROD induction of 16 PAHs (the US EPA Priority Pollutant PAHs) in H4IIE cells were applied to measure induction equivalency factors (IEFs). The concentration of the test compounds best fitting the linear part of the 3-MC dose–response curve was chosen to calculate the IEF of the test compounds. Relative induction potencies of PAHs in comparison with the potent inducer 3-MC were calculated by setting the IEF of 3-MC as 1.0. Chemical 3-MC equivalents (chemical MEQ) values of water samples were calculated by multiplying IEFs by the relative amount of the corresponding agonist on a weight basis.

$$Chemical\ MEQ = \sum_{i=1}^{n} c_i \times IEF_i\ (EROD)$$

Relative induction potencies and the MEQ quantify the EROD activity of water samples. All sample extracts were calibrated against the standard curve of 3-MC for calculation of MEQ in the extract. The concentration-response curves for EROD induction were drawn using computer-aided nonlinear regression as model equations. The biological 3-MC equivalents (bio-MEQ) values were calculated according to the method of Hanberg *et al.*[10] The MEQ concentration per treated concentration of water sample was then calculated based on a comparison of effective concentration values. The EROD activity yield experiments conducted in quadruplicate wells were repeated at least three times and statistical tests were performed on all data. Along with each set of extracts, appropriate standards were analyzed on the same day.

Data Analysis —— Sigma Plot software (Jandel Science Software, San Rafael, CA, U.S.A.) and Excel (Microsoft, NY, U.S.A.) are used to analyze data and used with Student's *t*-test for comparisons. Statistical differences between groups were determined using data expressed as means ± S.D. At least three determinations were carried out for each data point.

RESULTS

Concentration of PAHs in Water Samples

The 16 PAHs, *i.e.*, naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo [*a*]pyrene, dibenzo[*a*,*h*]anthracene, benzo[*g*,*h*,*i*] perylene, and indeno(1,2,3-*cd*)pyrene, detected in the water samples from four different sites are presented as each concentration and as MEQ concentration in Table 1. PAH concentrations in water samples ranged from 0.01 ng/l (dibenzo[*a*,*h*]anthracene) to 27.08 ng/l (naphthalene). The total amount of PAHs was high in downstream water samples. The difference in total PAHs concentrations between upstream and downstream water samples was two fold. It should be noted that there was a difference in the PAH content between upstream and downstream water.

MEQ and IEF values of 16 PAHs were calculated by applying a sigmoidal equation determined by EROD microbioassay. Relative induction potencies in comparison with that of 3-MC were calculated by setting the IEF of 3-MC as 1.0.

The chemical MEQ was calculated by multiplying IEFs by the relative amount of the corresponding agonist on a weight basis. The sum of calculated chemical MEQ values was two or three fold higher than the total PAH concentration.

Induction of CYP1A by Water Samples

EROD activities, which are due to the CYP1A isozyme in rat hepatoma cell line H4IIE and rainbow trout hepatoma cell line RTH-149, were measured. The EROD activity of standard chemicals [2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-MC] in the H4IIE cell line was greater than in RTH-149. As shown in Fig. 1, a positive correlation between these two cell lines was observed. Therefore the water samples from downstream and upstream sites of two rivers were studied for their effects on H4IIE as a sensitive cell line. The induction of EROD by different concentrations of water extracts taken from four different sites is shown Fig. 2. The Gab stream seems to be more highly polluted with CYP1A-inducers than the Mankyung River. The water quality was quantitatively assessed by comparing the EROD activities of water samples with that of the standard 3-MC of known concentration. We previously proposed that the MEQ concentration is useful to quantify the mutagenic potential of

Table 1. PAH Concentration (ng/l) of River Water Samples from the Four Different Sites Determined by GC-MS

	IEF	GBU		GBD		MKU		MKD	
		Conc.	Che-MEQ[a)]	Conc	Che-MEQ	Conc	Che-MEQ	Conc.	Che-MEQ
Naphthalene	0.030	27.08	0.81	20.09	0.60	0.43	0.01	0.30	0.01
Acenaphthylene	0.000	0.28	0.00	2.31	0.00	0.04	0.00	0.16	0.00
Acenaphthene	0.000	0.01	0.00	0.55	0.00	—	—	0.17	0.00
Fluorene	0.000	0.17	0.00	3.60	0.00	—	—	2.72	0.00
Phenathrene	0.002	1.65	0.00	5.59	0.01	1.85	0.00	4.11	0.01
Anthracene	0.001	0.06	0.00	0.28	0.00	0.76	0.00	1.86	0.00
Fluoroanthene	0.003	0.73	0.00	1.89	0.01	0.77	0.00	1.73	0.01
Pyrene	0.002	0.52	0.00	1.83	0.00	1.20	0.00	1.81	0.00
Benzo[*a*]anthracene	4.800	0.86	4.13	1.19	5.71	1.48	7.10	1.68	8.06
Chrysene	0.440	0.24	0.11	0.36	0.16	—	—	0.43	0.19
Benzo[*b*]fluoranthene	1.600	3.39	5.42	3.99	6.38	3.93	6.29	3.91	6.26
Benzo[*k*]fluoranthene	8.600	2.81	24.17	1.40	12.04	2.64	22.70	3.04	26.14
Benzo[*a*]pyrene	0.590	3.92	2.31	0.62	0.37	3.25	1.92	3.96	2.34
Indeno(1,2,3-*cd*)pyrene	1.600	3.33	5.33	2.82	4.51	3.37	5.39	1.72	2.75
Dibenzo[*a*,*h*]anthracene	4.800	2.35	11.28	0.01	0.05	0.11	0.53	2.32	11.14
Benzo[*g*,*h*,*i*]perlene	0.000	1.46	0.00	0.03	0.00	2.49	0.00	1.64	0.00
Total		48.86	53.56	46.56	29.84	22.32	43.96	31.56	56.91

a) Chemical 3-MC equivalent: Che-MEQ values were calculated by multiplying IEFs by the relative amount of the corresponding agonist on a weight basis. Sixteen carcinogenic PAHs (US EPA Priority Pollutant PAHs) were used to measure IEFs of EROD induction in H4IIE cells.

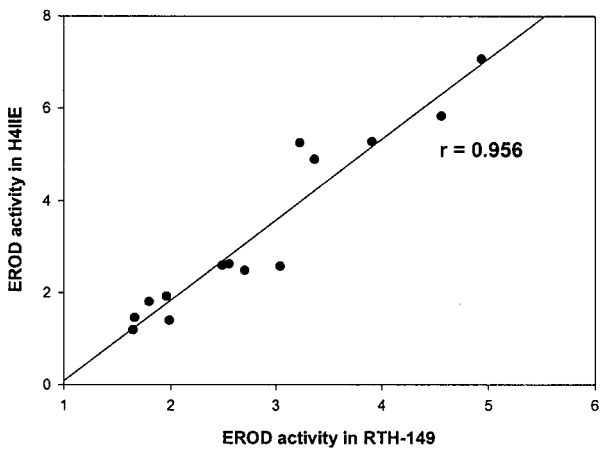

Fig. 1. Correlation between EROD Activity in RTH-149 and H4IIE Cells Treated with TCDD and 3-MC

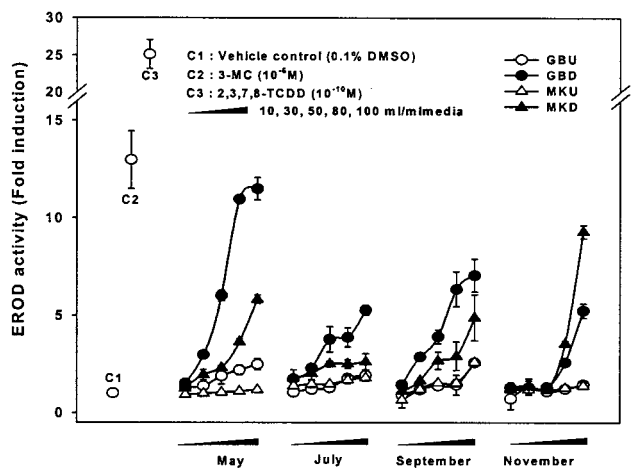

Fig. 2. Concentration-Response Curves for EROD Activity in H4IIE Cells Grown in 96-well Plates after 48-hr Incubation with River Water Samples from the Gab Stream (GB) and Mankyung River (MK) in Culture Medium with 5% fetal Bovine Serum

U, upstream; D, downstream.

water.[11)] In this study we defined two MEQ concentrations. One is the chemical MEQ calculated by multiplying the IEF and PAH concentration. The other is the bio-MEQ calculated by comparing the concentration-response curve of the sample with those of the 3-MC calibration curve. For calculating bio-MEQ, concentration-response curves of 3-MC and water samples were obtained using the EROD microbioassay and then normalized by expressing the activity values as percentages compared with that of 3-MC 1 μM. The curves were described by the following sigmoidal equation, where x represents the concentration of the inducer and y the relative catalytic activity.

$$y = y_0 + \frac{a}{1 + e^{(-\frac{x - x_0}{b})}}$$

Calculation was done by the iterative approxi-

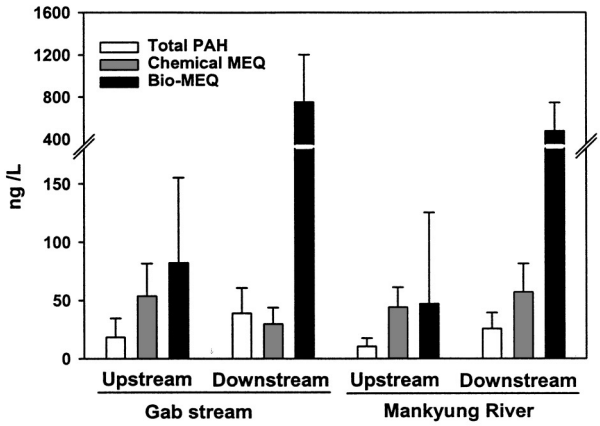

Fig. 3. Comparison of Total PAH Concentration, Chemical MEQ, and Bio-MEQ in Water Samples

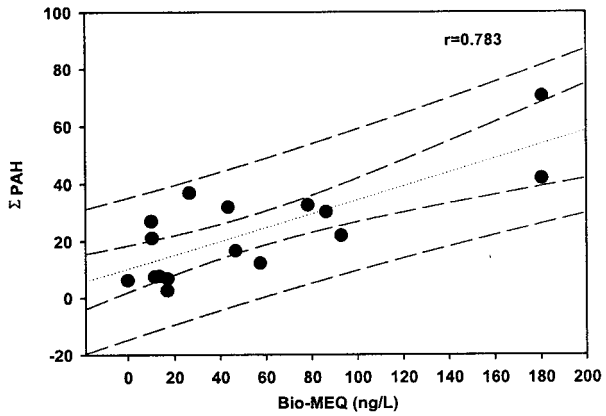

Fig. 4. Correlation between Bio-MEQ and Total PAH Concentration in Water Samples

mation of the constants a, b, x_0, y_0 with the computer program Sigma Plot software. The degree of curve fitting was indicated by the coefficient of correlation, which has the highest value of 1, when 100% fitting would be established. EROD activity of water samples was converted to MEQ concentration. As shown in Fig. 3, the bio-MEQ values of downstream water extracts were significantly higher than those of upstream water extracts. The difference between total PAH concentration and bio-MEQ was 20-fold in downstream water samples. However, in upstream water samples bio-MEQ was only five fold higher than the total PAH concentration.

DISCUSSION

The quantification of the total risk of toxicity becomes increasingly impossible due to the lack of instrumental analytical capabilities and missing models of action for mixtures. Fortunately, the response of compounds can be detected bioanalytically focusing on the source and matrices of concern. Such novel *in vitro* assays are a new tool to investigate lipophilic and low volatile compounds, they are powerful complements to instrumental analysis, offer an approach to discover unknown toxicants, are low cost and highly efficient, represent a scientific challenge to quantify endpoint-related risks, and therefore are a basis for effect-targeted risk assessment.

The EROD microbioassay was conducted to determine CYP1A activity in environmental samples and the calculated MEQ concentration was introduced as a new quantitative water quality parameter for PAHs. Chemical MEQ is an estimation of the toxic effects of PAHs and bio-MEQ estimates the total toxic effects of various CYP1A-inducing chemicals in water samples.

In this study, be bio-MEQ values of water samples were higher than chemical MEQ values and total PAH concentrations, indicating that there must be undetected compounds other than the 16 PAHs in the water samples as effective inducers of EROD and that the biological activities of mixture compounds are mainly due to additive effects. There was a good correlation between bio-MEQ and total PAH concentrations ($r = 0.783$, Fig. 4) and the difference between bio-MEQ and total PAH concentration was high in downstream, *i.e.*, polluted sites. High sensitivity was observed in the H4IIE cell line compared with the fish cell line RTH-149. We concluded from the experiment described above that analysis of only 16 PAHs does not give sufficient information about the biological potency of water samples. The measurement of bio-MEQ using the H4IIE EROD microbioassay is useful for the quantitative assessment of water quality containing PAH-like compounds.

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