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# Quantitative Assessment of Mutagenic Potential of Water via EROD-Microbioassessment

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Induction of cytochrome P450IA detected by 7-ethoxyresorufin-o-deethylase activity in the rat hepatoma cell line (EROD microbioassay) had been used qualitatively to detect the presence of environmentally harmful compounds such as polycyclic aromatic compounds (PAHs), biphenyl ethers, naphthalenes, polychlorinated biphenyls (PCBs), and dioxin-like compounds in stream water samples and fly ashes (Schwirzer et al. 1998). Most of these compounds are lipophilic and resistant to biochemical degradation. As a result, these chemicals accumulate in biological tissues of the food chain endangering the health of humans and ecosystems (Patterson et al. 1994). Without using the costly, time-consuming, and often-difficult chemical analysis of all these compounds, the EROD microbioassay offers a relatively quick and cost-effective way to detect the presence of these compounds. However useful as a primary screening tool, the EROD microbioassay has rarely been used to quantitatively assess various toxic potential such as weight loss, thymic atrophy, subcutaneous edema, immune suppression, and hormonal alterations due to presence of these pollutants.

In this paper, we determined the optimal separation/extraction methods using XAD-4 resin, optimal cell and incubation period for the EROD microbioassay, and introduced 3-methlychoranthrene (3-MC) equivalent concentration as a new quantitative water quality parameter.

## MATERIALS AND METHODS

Using the amberlite XAD-4 resin, the pollutants in water are separated by static adsorption, eluted in the soxhlet extractor, and concentrated in a rotary evaporator. To determine the optimal doses of the XAD-4 resin, the duration of adsorption periods, and the soxhlet extraction efficiencies, an adsorption-extraction experiment was performed with a model PAH compound, Benzo(a)pyrene (B(a)P). B(a)P is a known carcinogenic compound which could be used as an internal standard. In each 1 L of distilled water sample, 1 mL of $10^{-7}$ M B(a)P was added. The XAD-4 resin concentration was varied from 0.1 to 1.5 g/L. Two sorption periods were examined, i.e., 12 and 24 hours. B(a)P adsorbed to the resin was eluted with methylene chloride/ethyl acetate mixture (9:1) in a soxhlet extractor for 3 hr. The methylene chloride/ethyl acetate mixture was evaporated at low pressure. The residue was then redissolved in toluene and analyzed with GC-FID. B(a)P was separated in Ultra-2

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column (30 m x 0.32 mm, I.D. 0.25 μm $d_f$). The GC was maintained at 80 °C for 1 min, programmed at 30 °C/min to 290 °C (30 min at 290 °C) with an injector temperature 280 °C. The flame ionization detector (FID) was maintained at 290 °C.

To determine the cell type most sensitive to the EROD assessment, rat hepatoma cell line (H4IIE), mouse hepatoma cell line (Hepa1c1c7), and human hepatoma cell line (HepG2) were examined with 3-MC at concentrations ranging from $10^{-12}$ to $10^{-6}$ M. These cells for were obtained from the American Type Culture Collection.

Five liters of water samples were acidified to pH 3 with concentrated $H_2SO_4$ and stirred with 2.5 g Amberlite XAD-4 resin (Fluka) for 24 hours. The pollutants adsorbed to the resin were eluted with methylene chloride/ethyl acetate mixture (9:1) in soxhlet extractor for 3 hours. The methylene chloride/ethyl acetate mixture was then evaporated in a rotary evaporator. The residue was dissolved in 10% Dimethylsulfoxide (DMSO) and stored in –20 °C until the assays were conducted. The final solvent concentration in the medium did not exceed 0.1% (v/v).

Cells were grown in Eagle's minimum essential medium (MEM, Gibco) supplemented with 5% fetal bovine serum (FBS, Hyclone) in a humidified incubator at 37 °C and 5% $CO_2$/95% air. The EROD activity was measured as described by Drenth et al. (1998). The cells were plated at a density of 50,000 cells/ well on 24-well plates in MEM supplemented with 5% FBS. After cells were allowed to attach for 48 hr, the seeding medium was removed and replenished with the phenol-red free MEM/5% FBS. Five μL of the above water sample extract residues dissolved in 10 % DMSO was added to the 1 mL this phenol-red free MEM and incubated at 37 °C and 5% $CO_2$/95% air. After 48 hr of incubation, the cells were washed twice with the phosphate buffered saline (PBS) and added to phenol-free MEM containing 5 μM dicumarol and 4 μM ethoxyresorufin. The cells were incubated for 30 min at 37 °C and the medium was removed from the wells. The fluorescence of the medium was measured at 530/588 nm excitation/emission wavelengths. After the EROD activities were determined, the cells were lysed with 1 mL 0.1 M NaOH and the protein content of the wells was determined using the Lowry method (Lowry et al. 1951).

3-methylchoranthrene (3-MC, Aldrich), a common EROD inducer, was used as a standard for the estimation of EROD activity equivalency in H4IIE. The maximum induction response (MIR) and the 3-MC equivalent concentration (MEQ) quantify the EROD activity of water samples. MIR compared the maximal EROD activity induced by the dioxin-like pollutants in the water samples with that induced by 3-MC. MIR is calculated as:

$$MIR = \frac{100(FI-1)_{WaterSample}}{(FI-1)_{3\text{-}MC}} \qquad [1]$$

where, FI (fold induction) = ratio of the EROD activity of sample to that of the negative control. The 3-MC equivalent concentration (MEQ, ng 3-MC/L-sample) was calculated comparing the maximum effective concentration of the sample's dos-response curve with those of the 3-MC calibration curve. The EROD activity yield

experiments conducted in quadruplicate wells were repeated at least 3 times and statistical tests were performed on all data.

## RESULTS AND DISCUSSION

The adsorption characteristics of our model PAH compound, i.e., B(a)P, on the XAD-4 resin and the extraction efficiency of the soxhlet extractor are shown in Table 1. For the water samples with XAD-4 resin concentrations more than 0.5 g/L and 24 hr of adsorption time, almost 100% of the B(a)P in was separated and extracted. Our static adsorption method coupled with XAD-4 resin having larger surface area improved the separation and extraction efficiency than the popular dynamic adsorption method with XAD-2 resin.

**Table 1.** Separation/extraction efficiency of B(a)P with XAD-4 resin in static adsorption mode with soxhlet extraction.

| Adsorption time (hrs) | XAD-4 amount (g) | Peak area ratio (B(a)P/I.S.) | Recovery (%) |
|---|---|---|---|
| 12 | 0.1 | 0.104 | 4.1 |
| | 0.5 | 1.051 | 41.6 |
| | 1.0 | 1.966 | 77.8 |
| | 2.0 | 2.014 | 79.7 |
| 24 | 0.1 | 0.924 | 36.6 |
| | 0.5 | 2.665 | 105.5 |
| | 1.0 | 2.634 | 104.2 |
| | 2.0 | 2.562 | 101.4 |

I.S. : Internal Standard(docosane), B(a)P : Benzo(a)pyrene

Among the three cell lines tested, H4IIE cell line showed the highest EROD activity at high 3-MC concentrations (Figure 1). The EROD activity of Hepa1c1c7 decreased rapidly at 3-MC concentration above $10^{-7}$ M. It appeared to reach its toxic level above $10^{-7}$ M 3-MC. Therefore, H4IIE was selected for the rest of the EROD assays since it is sensitive at higher 3-MC concentrations.
The optimal incubation period was determined by incubating the H4IIE cell line at 3-MC concentration ranging from $10^{-10}$ to $10^{-6}$ M for 24, 48, and 72 hr. For 3-MC concentration less than $10^{-6}$ M, both 48 and 72 hr of incubation produced high EROD activities as shown in Figure 2. Although the samples incubated for 72 hr showed higher EROD activity at $10^{-6}$ M 3-MC concentration, 48 hr incubation period was selected since it is less likely to encounter water samples with such high 3-MC equivalent concentration and it would expedite the screening process.

The H4IIE cells were incubated with $10^{-6}$ M of 3-MC for 48 hr, and the formation of resorufin from 4 μM 7-ethoxyresorufin increased with reaction time and reached the highest activity at 30 min. The EROD activity gradually decreased after 30 min of reaction time. Therefore, the optimal reaction of 30 minutes was selected.

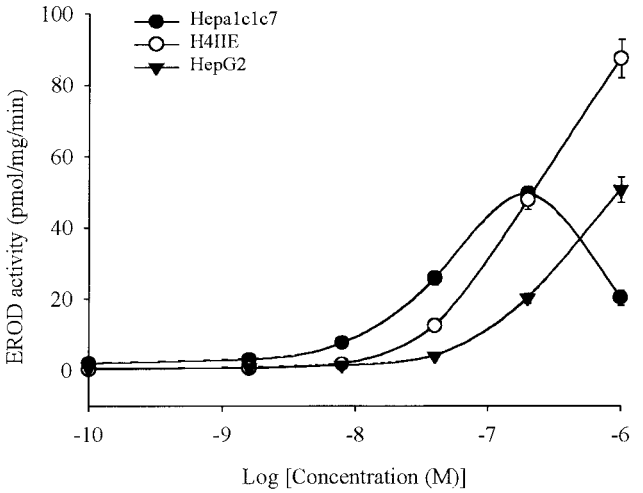


**Figure 1**. EROD activity with Hepa1c1c7, H4IIE, HepG2 cells.

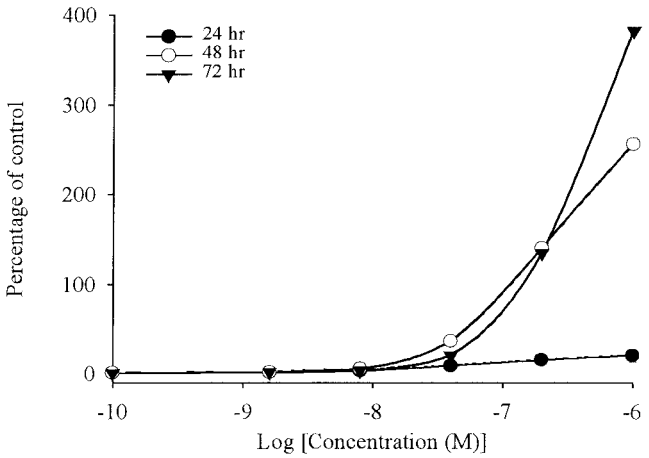


**Figure 2**. EROD activity with H4IIE cells at various 3-MC concentrations.

The field water samples were collected from Kumho River located in the mid-south of Korea. Since the level of pollution sensitive to the EROD test was not known, the pollutants in the original water samples were concentrated 1000, 5000, and 10,000 times which are 10mL, 50mL, and 100mL as water samples using the combined solid-phase extraction technique. Figure 3 shows the

dose-response characteristics of the water samples taken five different sites. The EROD activity increased with the concentration factor to 5,000 times (50 mL), but decreased sharply at 10,000 (100 mL), indicating that the pollutant concentration reached to the level inhibitory for the cytochrome P450 enzyme system. Therefore, the water samples were concentrated to 5,000 times for the subsequent quantitative assessment.

Water quality of the Kumho River was quantitatively assessed by comparing the EROD activities of water samples with that of the standard 3-MC with known concentration. The maximum induction response (MIR) was determined with the EROD activity at doses of 3-MC ranging from $10^{-10}$ M to $10^{-6}$ M as shown in Figure 2. The EROD activity was minimal until the 3-MC concentration reached to $10^{-9}$ M, then increased with 3-MC concentration to the peak at $10^{-7}$ M, and sharply decreased at $10^{-6}$ M. The EROD activity of the water samples were compared and expressed as % of MIR (i.e., the EROD activity at $10^{-7}$ M 3-MC solution). Table 2 shows the % MIR of the Kumho River taken at different sites and times.

**Table 2**. Percent maximum induction response of EROD activity of Kumho River (Percent of EROD activity at 0.1 μM MC)

| **Periods** | **June** | **July** | **August** | **October** |
|---|---|---|---|---|
| **Volume** | **50ml** | **50ml** | **50ml** | **50ml** |
| **KH-1** | 13.73 ± 0.46 | 11.11 ± 1.07 | 5.16 ± 0.16 | 7.55 ± 0.64 |
| **KH-2** | 27.23 ± 1.27 | 12.09 ± 2.42 | 4.03 ± 0.62 | 16.12 ± 0.79 |
| **KH-3** | 14.83 ± 0.06 [1)] | 13.65 ± 1.40 | 9.94 ± 0.62 | 23.90 ± 0.98 |
| **KH-4** | 15.57 ± 0.22 [1)] | 12.75 ± 0.27 | 13.73 ± 0.43 | 17.76 ± 0.26 |
| **KH-5** | 21.52 ± 0.07 | 12.25 ± 0.77 | 7.86 ± 0.44 | 1.29 ± 0.01 |
| **3-MC** | 100.00 ± 11.48 | | | |

[1)] Dilution volume treated cells : 10 mL

Although MIR had been used by Scholz and Segner (1999), it is not a very convenient unit for directly expressing the pollutant strengths. MIR is relative to the maximum concentration of 3-MC, which may be different for different cells or different incubation conditions, etc. Instead, we propose a new direct water quality parameter, 3-MC equivalent concentration (or 3-MEQ). We assumed a linear dose-response relationship exist between 3-MC concentrations of $10^{-8}$ M and $10^{-7}$ M. In this way, water quality with respect to the EROD microbioassay can be directly expressed as ng/L of 3-MEQ. The 3-MEQ is a gross water quality parameters similar to the chemical oxygen demand (COD) or biochemical oxygen demand (BOD). It can be used to directly express water quality of various water bodies and the efficiency of treatment performance.

Table 3 and Figure 4 show the 3-MEQ of the same Kumho River water samples as in Table 2. Both Figure 4 and Table 3 clearly show that the 3-MEQ concentrations of the samples KH-3 and KH-4 sharply decreased from June to July to October due

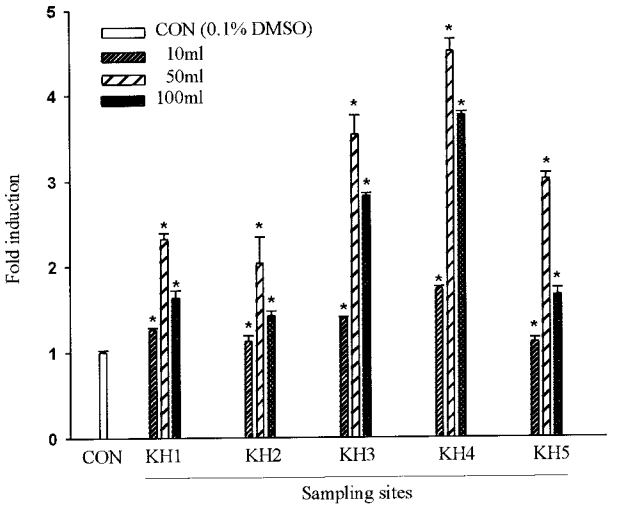


**Figure 3.** Dose-response EROD activity of Kumho River samples taken at different sites.

to dilution effect of water from precipitation. However, the sharp decrease in KH-3 and KH-4 sample in June and July is not readily evident in Table 2 because the % MIR for the samples were based on 10 mL water instead of 50 mL water sample. The KH-3 and KH-4 samples taken in June showed the MIR at 10 mL water sample instead of 50 mL as others. This clearly illustrates the advantage of using the 3-MEQ instead of %MIR to express the water quality relative to the EROD activity.

**Table 3**. 3-MEQ (3-MC equivalent concentration) of Kumho River (ng/L)

| Periods | June | July | August | October | Average |
|---|---|---|---|---|---|
| **KH-1** | 13.18 ± 0.44 | 11.74 ± 1.13 | 8.70 ± 0.26 | 9.77 ± 0.83 | 10.85 |
| **KH-2** | 25.10 ± 1.17 | 12.30 ± 2.47 | 8.31 ± 1.27 | 14.78 ± 0.72 | 15.12 |
| **KH-3** | 68.97 ± 0.29 | 13.17 ± 1.35 | 10.96 ± 0.68 | 21.37 ± 0.87 | 28.62 |
| **KH-4** | 72.24 ± 1.02 | 12.58 ± 0.27 | 13.17 ± 0.41 | 16.21 ± 0.23 | 28.55 |
| **KH-5** | 19.04 ± 0.06 | 12.30 ± 0.77 | 9.99 ± 0.56 | 7.24 ± 0.05 | 12.14 |
| **Average** | 39.71 | 12.42 | 10.23 | 13.87 | |

3-MC Concentration of Maximum induction response (100%) is 40.62 μg/L.

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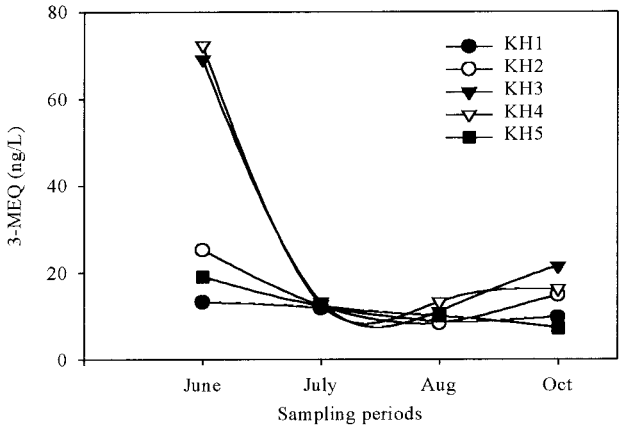


**Figure 4**. 3-MEQ values of Kumho River water samples.

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