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QSARs for Predicting the Toxicity of Mixtures Containing Polar Narcotic Chemicals

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It has been widely accepted that toxic effects are often generated by multiple mixtures rather by single chemicals in the environment.

Introduced by pharmacologists, the QSAR approach has emerged as one of the most promising methods of evaluating the toxicity for single chemicals provided that adequate physicochemical parameters of single chemicals (such as K_{OW}, α, β, E_{LUMO}, E_{HOMO} and so on) are known. Similarly, if these physicochemical parameters can be obtained, development of QSAR in the field of mixture toxicity will provide a good way to predict such toxicity. In order to assess the potential risks of mixture pollutants in the environment, Verhaar et al. (1995) first extended K_{OW} to mixtures and described the partition coefficient of a mixture as follows:

$$K_{MD} = \frac{W}{V} \times \frac{\sum_{i=1}^{n} \frac{Q^0_{water,i}}{1 + \frac{W}{VK_{SDi}}}}{\sum_{i=1}^{n} Q^0_{water,i} - \sum_{i=1}^{n} \frac{Q^0_{water,i}}{1 + \frac{W}{VK_{SDi}}}} \qquad (1)$$

Here K_{MD} is the C_{18}-Empore™ disk/water partition coefficient for a mixture. K_{SDi} is the partition coefficient of the individual chemical *i*. W is the volume of solution. V is the volume of the hydrophobic phase. Q^0_{water} is the initial amount of chemical *i* in water. *n* is the total number of individual chemicals in the mixture. The value of W/V is suggested as 6.8×10^5. According to the K_{MD} calculated from Eq.1, a K_{MD}-based QSAR model on mixture toxicity prediction was sucessfully proposed in our previous study (Lin et al. 2002). However it is only suitable for mixtures containing nonpolar narcotic chemicals, and therefore this model could not be generally used to predict the toxicity of environmental pollutants, as these pollutants may also contain polar narcotic chemicals, such as phenol, aniline and so on. Kamlet et al. (1986a) observed that the toxicity of individual polar narcotic chemicals is related not only with hydrophobicity but also hydrogen bond donor activity. Furthermore, he proved that the corresponding toxicity can be described by the octanol-water partition coefficient (Kow) and hydrogen donor acidity (α), respectively

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(Kamlet et al. 1986b, 1988). However, for mixtures containing polar narcotic chemicals, it still remains unclear how these hydrophobicity and hydrogen bonds work on organisms, and whether the overall hydrophobicity and the overall hydrogen bond activity in the mixture can be described by K_{MD} and α, respectively. These are exactly the questions we are attempting to answer in this study.

The purposes of this study, therefore, are: 1) to measure the toxicity of mixtures containing polar narcotic chemicals, 2) to observe the partitioning behavior of individual polar narcotic chemicals from the water phase to the biophase, and 3) to study how the hydrogen bond donor activity of individual chemicals contribute to mixture toxicity, thereby providing information for QSARs model to predict the toxicity of mixtures containing polar narcotic chemicals.

MATERIALS AND METHODS

Nitrobenzene, 3-bromo-nitrobenzene, phenol, 2,4-dichloro-phenol, aniline, 3-chloro-aniline and 3,4-chloro-aniline were purchased in the highest available purity from ACROS Organics Inc. The stock solutions for these chemicals were prepared in methanol (pro analysis grade), stored at –20℃ and used throughout this study. For testing, aliquots were evaporated under N_2 and redissolved in 3% NaCl solution.

The freeze-dried marine bacterium, *Vibrio fischeri* (T_3 mutation), was supplied by the Institute of Soil Science, Academic Sciences, Nanjing PRC. It was reconstituted and maintained on agar slants at 4℃. The bioluminescence assays were performed using diluted bacteria that had been cultured at 20℃ in yeast-tryptone-salts-gycerol broth for 12~14 h.

Toxicity was measured with a model toxicity analyzer DXY-2 (made by the Institute of Soil Science, Academic Sciences, Nanjing PRC) by quantifying the decrease in light emission from the bacteria as a result of exposure to 3% NaCl solution containing the test chemicals for 15 min. The decrease in light emission was measured at six different concentrations and each was tested in triplicate. Based on the decrease in light emission, the median effective concentration (EC_{50}) was calculated using the probit model (Finney 1971). The toxicity of 7 single chemicals was measured and reported as log $1/EC_{50}$ ($mol \cdot L^{-1}$). The mixture toxicity tests were conducted in a similar manner to the single chemical tests. It was assumed that the initial concentration of the mixture was 100%, the decrease in light emission was measured at six different concentrations, 10%, 18%, 32%, 56%, 80%, 100%, and therefore the median effective concentration was calculated in the unit of percentage (%). The mixture toxicity was quantitatively described as follows (Preston et al. 2000),

$$EC_{50M} = \frac{C_M}{\frac{C_A}{EC_{50A}} + \frac{C_B}{EC_{50B}} + \cdots} \qquad (2)$$

Here EC_{50} is the effective concentration required to bring about a 50% decrease in light output. C is the concentration of an individual chemical, and subscripts A, B, ……

and M are the individual chemicals and mixture, respectively. Because the joint effect of these polar narcotic chemicals are concentration additive, the concentration of the individual chemical (C_A, C_B, ……) could be calculated according to the median effective concentration in the unit of percentage (%), that is C_A, C_B, ……= percentage %×the initial concentration of individual chemical. Furthermore, the joint effect is described by the sum of toxic units (M) as follows,

$$M = \frac{z_1}{Z_1} + \frac{z_2}{Z_2} \qquad (3)$$

where z_i is the toxicant concentration C_A, C_B, ……, and Z_i is the EC_{50} value. Combining z_1 and z_2 resulted in an exact 50% response. Concentration addition is characterized by M=1, where M>1 represents antagonism and M<1 indicates synergism.

The partitioning behaviors of individual polar narcotic chemicals from the water phase to the biophase were studied by measurements of individual chemicals in cells of *Vibrio fischeri*. After exposure to individual chemicals or mixtures for 15 min, the 500 ml-bacteria cells were concentrated with a continuous flow centrifuge (Avanti^{TM} J-20XP, Beckman) at 10,000×g for 10 min (Nakamura and Matsuda 1971). To remove the polar narcotic chemicals on the surface of cells, these bacteria cells were washed with 100 ml-distilled water (dH_2O) and were re-concentrated by centrifugation at 10,000×g for 10 min. These washed cells were collected in a 10-ml tube and were given a final volume of 5 ml by using dH_2O. Then the 5 ml washed cells were disrupted in an ultrasonic disintegrator five times, each disrupting process lasted 1 min with a 1 min break in between. During this handling, the temperature was kept at 0℃ by immersing the sample in a water-ice bath. After this handling, the individual chemicals in these cells were extracted with 2 ml n-hexane by ultrasonic cleaning for 20 min. Finally, this n-hexane extract was filtered with the 0.45 μm organic film and analyzed by GC (HP6890) as follows: 1.0 μL injection for each sample at 240℃, with the detector at 280℃. The column temperature was started at 30℃, then increased to 260℃ at a rate of 20℃/min, and then held at 260℃ for 8.5 min. The partitioning amount of chemicals from the water phase to the biophase was then calculated by comparing the integrated intensity of the GC peaks with that of authentic samples.

The octanol-water partition coefficients (K_{OW}) were calculated by the fragment constant methodology of Hansch and Leo (1979). According to Verhaar et al. (1995), the correlation between log K_{OW} and log K_{SD} (C_{18}-Empore^{TM} disk/water partition coefficient of single chemicals) was described by Eq. 4 (n=18, r^2=0.93, SE=0.24).

$$\log K_{SD} = 0.995 \log K_{OW} + 0.70 \qquad (4)$$

The log K_{SD} of the single chemicals studied were calculated (Eq. 4 and Table 1). Furthermore, log K_{MD} values were obtained by using Eq. 1 (Table 2, Table 3).

RESULTS AND DISCUSSION

The toxicity of 7 individual chemicals and 20 mixtures to *Vibrio fischeri* was determined and the results are given in Table 1 and Table 2, respectively. As shown in Table 2, for mixtures containing polar narcotic chemicals, the concentration addition occurred, with M ranging from 0.85 to 1.15. The same overall results were observed in the study of EIFAC (1987), Könemann (1981) and Hermens (1989). Therefore, this concentration addition indicates that, similar to that of single polar narcotic chemicals, the toxicity of mixtures containing polar narcotic chemicals is correlated primarily with their partitioning behaviors and their hydrogen bond donor activity.

The mixture partitioning behaviors from the water phase to the biophase were studied by measurements of individual chemicals in cells of *Vibrio fischeri*. Since concentration addition is found for polar narcotic chemicals, the concentration of mixtures can be assumed as the total molar concentration of individual chemicals (Verhaar et al. 1995). Based on this assumption and the equilibrium partition models (EPMs), the partitioning behaviors of chemical mixtures from the water phase to the biophase (K_{real}) can be described as follows,

$$K_{real} = \frac{C^t_{cell}}{C^t_{water}} = \frac{\frac{Q^t_{cell}}{V_{cell}}}{\frac{Q^t_{water}}{V_{water}}} = \frac{V_{water}}{V_{cell}} \times \frac{Q^t_{cell}}{Q^0_{water} - Q^t_{cell}} \qquad (5)$$

where, C^t_{cell} is the total concentration of chemicals partitioning from water to cell at equilibrium time. Q^t_{cell} is the total amount of chemicals partitioning from water to cell at equilibrium time. C^t_{water} is the total concentration of the mixture in water at equilibrium time. Q^t_{water} is the total amount of the mixture in water at equilibrium time. V_{water} is the volume of water. V_{cell} is the volume of cell. Q^0_{water} is the initial amount of the mixture in water.

Table 1. Partition coefficients and toxicity for single polar narcotic chemicals

No	Individual Chemicals	$\log K_{OW}$	$\log K_{SD}$	$\log 1/EC_{50}$ [mol·L^{-1}]	$\log 1/(EC_{50})_{baseline}$ [mol·L^{-1}]	$\log 1/(EC_{50})_{hydrogen}$ [mol·L^{-1}]
#1	nitrobenzene	1.87	2.56	3.22	2.65	2.79
#2	3-bromo-nitrobenzene	2.69	3.38	4.18	3.38	3.45
#3	phenol	1.46	2.15	2.95	2.29	2.40
#4	2,4-chloro-phenol	2.89	3.58	4.64	3.56	3.60
#5	aniline	0.92	1.62	2.29	1.82	1.99
#6	3-chloro-aniline	1.63	2.32	3.57	2.44	2.47
#7	3,4-chloro-aniline	2.32	3.01	4.20	3.05	3.08

Table 2. Partition coefficients, hydrogen bonding effect and toxicity for mixtures containing polar narcotic chemicals

No.	Individual chemicals in mixtures	$-\log[\Sigma P_i*(EC_{50i})_{hydrogen}]$ [mol·L^{-1}]	log K_{MD}	M	$\log 1/EC_{50M}$ [mol·L^{-1}]	$\log 1/(EC_{50M})_{baseline}$ [mol·L^{-1}]	$\log 1/(EC_{50M})_{hydrogen}$ [mol·L^{-1}]
1	#1: #2	2.82	2.75	0.99	3.48	2.78	2.87
2	#1: #3	2.50	2.34	0.86	3.13	2.40	2.48
3	#1: #4	2.80	2.69	0.85	3.58	2.72	2.78
4	#1: #5	2.00	1.88	1.03	2.53	1.97	2.11
5	#1: #7	2.81	2.63	0.91	3.52	2.66	2.73
6	#2: #3	2.42	2.43	0.85	3.30	2.48	2.55
7	#2: #4	3.48	3.44	1.05	4.33	3.42	3.47
8	#2: #5	1.97	1.85	0.97	2.60	1.94	2.05
9	#2: #6	2.55	2.81	0.85	3.85	2.83	2.88
10	#2: #7	3.23	3.24	0.98	4.20	3.23	3.28
11	#3: #4	2.41	2.33	1.15	3.17	2.39	2.46
12	#3: #5	2.01	1.77	1.01	2.50	1.87	1.98
13	#3: #6	2.41	2.19	1.04	3.14	2.26	2.32
14	#3: #7	2.42	2.27	1.15	3.16	2.33	2.40
15	#4: #5	1.96	1.77	0.98	2.60	1.87	1.96
16	#4: #6	2.50	2.69	0.90	3.88	2.72	2.75
17	#4: #7	3.17	3.25	0.85	4.44	3.24	3.27
18	#5: #6	1.98	1.70	0.87	2.63	1.80	1.87
19	#5: #7	1.96	1.73	1.12	2.50	1.83	1.93
20	#6: #7	2.54	2.56	0.85	3.85	2.60	2.62

Table 3. Partitioning amount of chemical mixtures from the water phase to the biophase

Mixtures	Initial amount of mixture in water, Q^0_{water} ($\times 10^{-5}$mol)	Total amount of mixture in cell, Q^t_{cell} ($\times 10^{-8}$mol)	log K_{real}	log K_{MD}
#1	45.630	4.037	-4.05	2.56
#2	4.955	23.176	-2.33	3.38
#6	20.187	2.213	-3.96	2.32
#7	4.678	6.221	-2.88	3.01
#1+#2	#1: 22.82 #2: 2.478	1.825 10.430	-3.32	2.75
#1+#6	#1: 22.82 #6: 10.094	2.132 1.262	-3.98	2.50
#1+#7	#1: 22.82 #7: 2.339	2.965 3.080	-3.62	2.63
#2+#6	#2: 2.478 #6: 10.094	10.072 1.148	-3.05	2.81
#2+#7	#2: 2.478 #7: 2.339	10.980 3.892	-2.51	3.24
#6+#7	#6: 10.094 #7: 2.339	0.954 3.380	-3.46	2.56
#1+#2+#6	#1:22.82 #2:2.478 #6:10.094	1.430 10.883 3.078	-3.61	2.66
#2+#6+#7	#2:2.478 #6:10.094 #7:2.339	10.045 1.112 3.573	-3.01	2.85

Since $Q^0_{water} \gg Q^t_{cell}$, Eq. 5 can be rewritten as,

$$K_{real} = \frac{V_{water}}{V_{cell}} \times \frac{Q^t_{cell}}{Q^0_{water}} \qquad (6)$$

In this case, the V_{water}/V_{cell} is a fixed value, so reduction of Eq. 6 gives,

$$K_{real} = \frac{Q^t_{cell}}{Q^0_{water}} \qquad (7)$$

The log K_{real} were calculated using Eq. 7 and the results are listed in Table 3. Furthermore, log K_{MD} values of the mixtures were also calculated using Eq.1 (Table 3). In finding the relationship between log K_{MD} and log K_{real}, regression analysis is carried out,

$$\log K_{real} = -8.440 + 1.862 \log K_{MD} \qquad (8)$$

n=8, r^2=0.893, SE=0.161, F=50.212, P=0.000

The significant correlation coefficient in Eq. 8 (r^2=0.893) shows a good consistency between K_{MD} and K_{real}. This consistency indicates that, for mixtures containing polar narcotic chemicals, their partitioning behaviors from the water phase to the

biophase can be described by K_{MD}. Hence, the toxicity contributed by this partitioning behavior can be obtained by the K_{MD}-based QSAR approach (Lin et al. 2002). In this study, this toxicity is defined as the baseline toxicity of mixture.

The hydrogen bond donor activity in mixtures containing polar narcotic chemicals is also revealed in this study. In our previous study (Lin et al. 2002), the baseline toxicity of single chemicals and that of mixture was quantified by Eq. 9 and Eq.10, respectively.

$$\log 1/EC_{50} = 0.880 \log K_{SD} + 0.401 \quad (9)$$
$$n=8,\ r^2=0.934,\ SE=0.194,\ F=84.726,\ p=0.000$$

$$\log 1/EC_{50M} = 0.928 \log K_{MD} + 0.224 \quad (10)$$
$$n=74,\ r^2=0.953,\ SE=0.130,\ F=1461.932,\ p=0.000$$

As mentioned above, for single polar narcotic chemicals, the toxicity (EC_{50}) is related to not only the partitioning of chemicals from the water phase to the biophase [$(EC_{50})_{baseline}$], but also the interaction between the hydrogen bonding groups in chemicals and that in biological macromolecules (Kamlet et al. 1986a) [$(EC_{50})_{hydrogen}$], which gives

$$(EC_{50})_{hydrogen} = (EC_{50})_{baseline} - (EC_{50}) \quad (11)$$

and concentration addition allows the generalization of Eq. 11 to the mixtures,

$$(EC_{50M})_{hydrogen} = (EC_{50M})_{baseline} - (EC_{50M}) \quad (12)$$

The baseline toxicity of single polar narcotic chemicals and that of the mixtures were obtained by using Eq. 9 and Eq. 10. Furthermore, based on these baseline toxicity data, the $(EC_{50})_{hydrogen}$ and $(EC_{50M})_{hydrogen}$ were calculated by using Eq. 11 (Table 1) and Eq. 12 (Table 2), respectively. To find the relationship between the $(EC_{50})_{hydrogen}$ and $(EC_{50M})_{hydrogen}$, regression analysis was carried out,

$$\log 1/(EC_{50M})_{hydrogen} = 1.022 \times \log 1/[P_i \times (EC_{50i})_{hydrogen}] - 0.015 \quad (13)$$
$$n=20,\ r^2=0.948,\ SE=0.112,\ F=325.247,\ P=0.000$$

where P_i is the molarity percentage of the individual chemical *i* in the mixture. It can be seen from Eq. 13 that, the slope (1.022) and the intercept (-0.015) are close to 1 and 0, respectively. This indicates that $(EC_{50M})_{hydrogen}$ represents the concentration addition of $(EC_{50})_{hydrogen}$.

In conclusion, for mixtures containing polar narcotic chemicals, the partitioning behaviors from the water phase to the biophase can be described by the C_{18}-Empore™ disk/water partition coefficient for a mixture (K_{MD}) and the baseline toxicity can be obtained by the K_{MD}-based QSAR approach. Furthermore, since concentration addition was found for $(EC_{50})_{hydrogen}$, it is assumed that the parameters (such as hydrogen donor α and hydrogen acceptor β), which describe the hydrogen bond donor activity in single polar narcotic chemicals, can be extended to the field of mixtures. Such parameters and the corresponding QSAR approach are proposed to predict mixture toxicity in a practical setting (Lin et al. 2003).

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