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Use of the IC_{50} for Predicting Joint Toxic Effects of Mixtures

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It has been generally accepted that the toxic effects are usually generated by multiple mixtures rather by single chemicals in the environment. And the research on mixture toxicity is booming in the past several decades. But so far, most of their studies only focus on developing the rational methodologies to classify joint effects as synergism, antagonism or addition (synergism occurs when one chemical increases the toxicity of another. Antagonism is the opposite effect: one chemical diminishes the toxicity of another. Addition represents that the mixture toxicity is the sum of the individual chemical toxicity), while few studies discuss how to predict the joint toxic effects (Chen and Chiou 1995, Xu and Nirmalakhandan 1998, Preston et al. 2000). Furthermore, the predicted results from these studies are insufficient for accurately predicting synergism or antagonism, as these studies are principally based on additive concept (Yang et al.1995). Therefore, a general approach for predicting any joint effects (synergism, antagonism or addition) is necessary.

As pointed out by toxicologists, chemicals occurring as complex mixtures have the potential for interactions which include chemical-chemical, toxicokinetic and toxicodynamic interactions, and these interactions have the potential to result in the joint toxic effects (Cassee et al. 1998, Steevens and Benson 2000). Chemical-chemical interactions are caused by the formation of products through chemical reactions between two or more parent chemicals in the mixture. Toxicokinetic interactions result from the alteration of chemical absorption, elimination, and distribution. Toxicodynamic interactions arise from the competition between individual chemicals for binding sites in an enzyme or other macromolecule. Apparently, toxicokinetic interactions are not directly related with enzyme activity, but the other two types of interactions do influence enzyme activity, either by formation of products or by competition for enzyme sites. Hence, given that some enzyme activity-based parameters for chemical mixtures can be found, the joint toxic effects resulted from chemical-chemical and toxicodynamic interactions will be predicted directly and accurately. For single chemicals, such a parameter was proposed by Dixon and Webb (1958) since most organic chemicals act as toxicants via inhibition of enzyme activity. They observed that, if the inhibition of an enzymatic reaction can be calculated as follows,

$$\%\text{inhibition} = \frac{\text{Activity}_{\text{control}} - \text{Activity}_{\text{test}}}{\text{Activity}_{\text{control}}} \times 100\% \qquad (1)$$

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an exponential curve will be found in the plot of the % inhibition versus toxicant concentration. Based on this exponential curve, they calculated the toxicant concentration that reduces enzyme activity by 50%, and then defined it as the IC_{50}. Furthermore, they proved that there is a significant relationship between the IC_{50} and the toxicity of the single organic chemicals. Therefore, if the IC_{50} can be applied in the field of mixtures, a correlation between the IC_{50} and the joint toxic effects would be found, and the joint toxic effects could be predicted directly on the basis of the correlation.

In this study, the bioluminescence properties of *Vibrio fischeri* are employed in a direct toxicity test, for numerous studies have shown that this test procedure is rapid, simple to perform, comparable to other environmental toxicity tests, low-cost, and sensitive to a multitude of chemicals (Lange and Thomulka, 1997). Furthermore, luciferase, an enzyme with which *Vibrio fischeri* generates light, is employed in the enzyme activity inhibition (IC_{50}) test. The objectives of this study are: 1) to determine the mixture toxicity to *Vibrio fischeri*, 2) to determine the IC_{50} to luciferase and extend it to the field of mixtures, and 3) to find out the relationship between the joint toxic effects and the IC_{50}, thus proposing an IC_{50}-based approach to predict the joint toxic effects.

MATERIALS AND METHODS

All 19 chemicals were purchased in the highest available purity from ACROS Organics Inc and are listed in Table 1. The stock solutions for these chemicals were prepared in methanol (pro analysis grade), stored at –20℃ and used throughout the study. For IC_{50} experiments, aliquots were evaporated under N_2 and redissolved in Sørensen buffer (pH 6.81). For toxicity experiments, aliquots were evaporated under N_2 and redissolved in 3% NaCl solution.

Mercaptoethanol, NADH (nicotinamide adenine dinucleotide, reduced form) and FMN (flavin mononucleotide) were obtained from SIGMA and freshly prepared in double-distilled water. To stabilize the enzyme activity, luciferase was dissolved in Sørensen buffer (pH 6.81). Decaldehyde was prepared in methanol because it may undergo autoxidation under aqueous solution (Watanabe and Nakamura 1972).

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 the diluted bacteria that had been cultured at 20℃ in 0.5%yeast-0.5%tryptone-3%salts-0.3%glycerol broth for 12~14 h.

Toxicity experiments were carried out with a toxicity analyzer (DXY-2, made by the Institute of Soil Science, Academic Sciences, Nanjing PRC). Before determination of toxicity, preliminary tests were used to determine the concentration ranges needed for testing. Toxicity was determined by quantifying the decrease in light emission from the bacteria as a result of exposure to test chemical solutions for 15 min. The decrease in light emission was measured at six

different test concentrations, with each was tested in triplicate. Then a linear regression for the decrease in light emission was drawn for these concentrations. Based on this linear regression, the EC_{50} (the effective toxicant concentration that causes the 50% decrease in light emission) was calculated using a linear interpolation method. The toxicity of 19 single chemicals was determined and reported as log ($1/EC_{50}$) in unit of $mol \cdot L^{-1}$ (Table 1). The mixture toxicity tests were conducted in a manner similar to the single chemical tests: The mixture is assumed as a special solute whose concentration is 100%. The decrease in light emission was measured at six different concentrations, 10%, 18%, 32%, 56%, 80%, 100%, and therefore EC_{50} was calculated in the unit of percentage (%) as p %. The joint toxic effect is described by the sum of toxic units (TU) as follows,

$$TU = \frac{z_1}{Z_1} + \frac{z_2}{Z_2} \qquad (2)$$

where Z_i is the EC_{50} value. z_i is the toxicant concentration and it is calculated according to p%, that is $z_i = p\% \times$ the initial concentration of individual chemical. Combining z_1 and z_2 results in an exact 50% response. The TU criterion for the mixtures to be concentration addition, synergism and antagonism is as follows: If the TU value is near 1.00 and its 95% confidence interval overlap 1.00, the mixture is concentration addition; If the TU value and its 95% confidence interval are greater than and do not overlap 1.00, then the mixture is antagonism; If the TU value and its 95% confidence interval are less than and do not overlap 1.00, then the mixture is synergism. The TUs and their 95% confidence interval of mixtures are given (Table 2).

The luciferase activity inhibition test (IC_{50}) was carried out on the basis of the luciferase-mediated enzyme reaction (Eq. 3) as follows,

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} FMN + H_2O + RCOOH + light \qquad (3)$$

where $FMNH_2$ is the coenzyme for luciferase, and in the test it is obtained from the dehydrogenase reactions (Eq. 4~5), because $FMNH_2$ is known to be rapidly oxidized by O_2 in air.

$$NAD(P) + RH_2 \longrightarrow NADPH_2 + R \qquad (4)$$

$$NAD(P)H_2 + FMN \longrightarrow NAD(P) + FMNH_2 \qquad (5)$$

It can be seen from Eq. 3 that, on the fixed condition, the luciferase activity determines the quantity of light emission. Therefore, the assay of luciferase activity was based upon the measurement of the light emission (Yoshida and Nakamura 1973). In this study, the light emission was measured by using a Flow Injection Chemiluminescence Analysis system (IFFM-D, made by Reman Electronic Science-Tech Co, LTD Xian PRC), and the specified IC_{50} test process was as follows: The solution containing 0.01% Mercaptoethanol (0.05 mL), pH 6.81 Sørensen buffer (1.00 mL), 13 mM NADH (0.20 mL), 0.42 mM FMN (0.20 mL) and toxicants (0.30 mL) was placed in a cuvette. This mixture solution was kept at 18 ± 0.1 ℃ in a water bath for 2 min. The second solution (0.2 mL of 10mg/mL luciferase) was placed in one syringe and the third solution (0.05 mL of 0.1% decaldehyde) was placed in the other syringe. The reaction was initiated by mixing these three solutions, and the light emission (λ_{max}=485 nm) from the reaction mixture was recorded. The light emission was measured at five different toxicant concentrations and each was

tested in triplicate. According to Eq. 1, the inhibition in light emission was calculated and then the IC_{50} (the concentration causing 50% inhibition) was calculated using a linear interpolation method. The IC_{50} of 18 individual chemicals are reported in Table 1. The inhibition of mixtures was conducted in a manner similar to the single chemicals tests. To qualitatively describe this mixture inhibition, the inhibition units (IU), similar to toxic units (TU), is proposed as follows,

$$IU = \frac{z_1}{Z_1} + \frac{z_2}{Z_2} \qquad (6)$$

where z_i is the inhibitor concentration and Z_i is the IC_{50} value. The IUs of mixtures are given (Table 2).

Statistical analyses were performed using the SPSS 9.0 software (SPSS Inc.). Model adequacy was measured as square of correlation coefficient (r^2), standard error (SE), F-ratio, and P value.

RESULTS AND DISCUSSION

Since Chen and Huang (1996) observed that cyanogenic toxicants have a high tendency to react via synergism with other reactive chemicals, malononitrile, α -hydroxy-isobutyronitrile, allyl cyanide, phthalic nitrile, benzonitrile, phenylacetonitrile and 12 other reactive chemicals were selected in this study. Their toxicity (EC_{50}) and IC_{50} were observed (Table 1). The relationship between EC_{50} and IC_{50} can be drawn as follows,

$$\log 1/EC_{50} = 0.259 + 0.954 \log 1/IC_{50} \qquad (7)$$
$$n=18,\ r^2=0.858,\ SE=0.434,\ F=96.691,\ P=0.000$$

The significant correlation coefficient in Eq.7 (r^2=0.858) shows a good consistency between log $1/EC_{50}$ and log $1/IC_{50}$. This consistency indicates that, for these reactive chemicals, it is primarily their inhibition of luciferase activity that leads to their toxicity to *Vibrio fischeri*.

Based on the observed EC_{50}, the toxicological joint effect between these cyanogenic toxicants and other reactive chemicals were determined and the results (TU) are listed in Table 2. It can be seen from Table 2 that the joint effect between malononitrile and acraldehyde, acetaldehyde, terephthalic aldehyde or *p*-nitrobenzaldehyde are all synergistic, with TU varying from 0.11~0.58 (No.1~8). Furthermore, this synergistic effect is independent of the proportions of individual chemicals. As shown by No.4~8 in Table 2, for mixtures containing malononitrile and *p*-nitrobenzaldehyde, although a change of proportions from 20:1 to 1:20 leads to a corresponding change in TU from 0.23 to 0.58, the TUs are always far less than 1 i.e. all the joint effects are synergistic. Chen and Huang (1996) attributed this synergistic effect to the formation of more toxic products through chemical-chemical interactions between the two parent compounds in the mixture. In this study these products were observed through the alteration of colorless solution to yellowish one, when $10EC_{50}$ *p*-nitrobenzaldehyde was mixed up with $10EC_{50}$ malononitrile. In spite of the formation of these products, the joint toxic effect for mixtures (including parent chemicals and their products) will correlate with their corresponding luciferase activity inhibition, provided that the products present their toxicity via their luciferase activity inhibition.

Table 1. Toxicity and IC_{50} of 19 individual chemicals

No.	individual chemicals	log $1/IC_{50}$ [mol·L^{-1}]	log $1/EC_{50}$ [mol·L^{-1}]
1#	malononitrile	2.40	2.55
2#	allyl cyanide	2.09	1.45
3#	phthalic nitrile	4.07	3.51
4#	benzonitrile	3.28	3.48
5#	phenylacetonitrile	5.03	4.23
6#	α -Hydroxy-isobutyronitrile	3.51	3.61
7#	acraldehyde	5.64	5.45
8#	acetaldehyde	2.22	2.36
9#	*n*-enanthaldehyde	3.72	4.05
10#	benzaldehyde	3.10	3.43
11#	terephthalic aldehyde	4.40	4.31
12#	*p*-nitrobenzaldehyde	4.31	4.28
13#	*p*-chlorobenzaldehyde	3.98	4.25
14#	paradimethylaminobenzaldehyde	5.39	5.88
15#	*p*-methoxybenzaldehyde	3.22	4.03
16#	*p*-nitroaniline	4.58	4.66
17#	*p*-dinitrobenzene	5.43	5.64
18#	*m*-dinitrobenzene	3.37	4.04
19#	β -hydroxypropyl-cycoldextrin	...	...

However, as shown in Table 2, there are additive effect for other mixtures containing cyanogenic toxicants and aldehydes (No.9~14). The same result was found by Chen and Huang (1996) and they proposed that this additive effect could be the net effect of two actions, a synergistic effect caused by the formation of more toxic products mentioned above and an antagonistic effect caused by "the complex joint action", which was defined in their study (Chen and Chiou 1995). In addition, the additive effect is observed for mixtures containing *p*-nitroaniline and *p*-dinitrobenzene, *m*-dinitrobenzene (No.15~17 in Table 2), and this additive effect is caused differently with No.9~14. It is caused by the non-interaction between individual chemicals because each of these chemicals acts in the same way, is described by the same QSAR model, and differs only in their potencies (Calamant and Vighl 1992, Zhao and Wang 1995, Groten 2000). Due to non-interaction, by now it has been a general conclusion that the enzyme activity inhibition from these chemical mixtures is the simple summation of that from individual chemicals.

At last, the antagonistic effect is found in Table 2 (No.18~20) for *p*-nitrobenzaldehydes in the presence β -hydroxypropyl-cycoldextrin. The reason for this antagonistic effect is that cycoldextrins (CDs) can include some organic chemicals by forming the host/guest inclusion complex, and therefore prevent the chemical active groups from the interaction of the enzyme-binding site.

Table 2. Joint effects and inhibition units for mixtures

No	Individual chemicals in the mixture	xEC_{50}: yEC_{50}	IU***	TU***			Joint effect
				Obs.	Pre.	Diff.	
1	1#: 7#	1:1	0.58	0.35 (0.29-0.41)*	0.44	-0.09	synergi
2	1#: 8#	1:1	0.41	0.11 (0.07-0.15)*	0.29	-0.18	stic
3	1#: 11#	1:1	0.50	0.18 (0.15-0.21)*	0.37	-0.19	
4		20:1	0.81	0.58 (0.50-0.66)*	0.64	-0.06	
5		10:1	0.52	0.58 (0.52-0.64)*	0.39	0.19	
6	1#: 12#	1:1	0.30	0.23 (0.18-0.28)*	0.20	0.03	
7		1:10	0.43	0.35 (0.27-0.43)*	0.31	0.04	
8		1:20	0.23	0.41 (0.35-0.47)*	0.14	0.27	
9	1#: 13#	1:1	1.23	1.08 (0.97-1.19)*	1.01	0.07	additive
10	1#: 14#	1:1	1.28	1.17 (0.96-1.38)*	1.05	0.12	
11	1#: 9#	1:1	1.00	0.76 (0.50-1.02)*	0.81	-0.05	
12	2#: 8#	1:1	1.11	1.03 (0.93-1.13)*	0.91	0.12	
13	3#: 8#	1:1	1.28	1.20 (0.98-1.42)*	1.05	0.15	
14	5#: 8#	1:1	1.12	0.93 (0.82-1.03)*	0.91	0.02	
15	16#: 17#	1:1	1.25	1.05 (0.85-1.25)*	1.03	0.02	
16	16#: 18#	1:1	1.32	0.94 (0.80-1.08)*	1.09	-0.15	
17	17#: 18#	1:1	1.28	0.85 (0.67-1.03)*	1.05	-0.20	
18		1‰**	1.58	1.25 (1.08-1.42)*	1.32	-0.07	antagon
19	19#: 12#	10‰**	2.02	1.57 (1.42-1.72)*	1.70	-0.13	istic
20		50‰**	3.20	2.85 (2.67-3.03)*	2.73	0.12	

*Note: Confidence intervals (95%) of TU

**Note: the percentage concentration of β -hydroxypropyl-cycoldextrin in solution.

***Note: Because of the less toxicity of β -hydroxypropyl-cycoldextrin, the EC_{50} of β -hydroxypropyl-cycoldextrin is so great that it will be enormously more than the concentration of β -hydroxypropyl-cycoldextrin in mixtures (C_B) i.e. $C_B \ll EC_{50B}$, and then the value of TU can be calculated as

$$TU = \frac{C_A}{EC_{50A}} + \frac{C_B}{EC_{50B}} = \frac{C_A}{EC_{50A}}$$

Table 3. Observed and predicted toxic units of 7 other related mixtures.

No	Individual chemicals in the mixture	xEC_{50}: yEC_{50}	IU	TU		
				Obs.	Pre.	Diff.
1	1#: 10#	1:1	0.80	0.63 (0.56-0.70)**	0.64	-0.01
2	1#: 12#	5:1	0.40	0.38 (0.28-0.48)**	0.29	0.09
3	1#: 15#	1:1	1.44	1.07 (0.92-1.22)**	1.19	-0.12
4	4#: 8#	1:1	1.13	1.00 (0.88-1.12)**	0.92	0.08
5	1#: 2#	1:1	1.30	1.05* (0.92-1.24)**	1.07	-0.02
6	6#: 7#	1:1	0.70	0.62* (0.48-0.75)**	0.55	0.07
7	6#: 8#	1:1	1.21	0.89* (0.70-1.14)**	0.99	-0.10

*Note: From Chen and Huang (1996)

**Note: Confidence intervals (95%) of TU

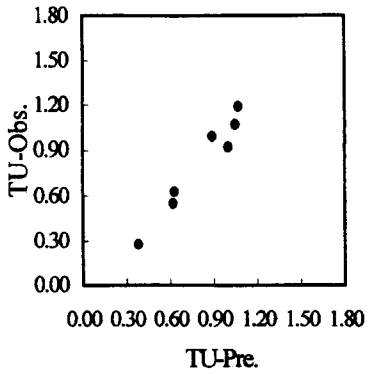

Figure 1. Observed toxic units versus predicted ones for 7 other related mixtures

As mentioned above, for these mixtures, whether the addition, synergism or antagonistic effect occurs, the mixture toxicity is highly correlated with their enzyme activity inhibition. To further study this correlation, the inhibitions of mixtures were also determined based on these IC_{50}, and the results (IU) are listed in Table 2. Using these IU, a linear regression on the TU is obtained,

$$TU = -0.064 + 0.874IU \qquad (8)$$
$$n=20,\ r^2=0.951,\ SE=0.140,\ F=350.802,\ p=0.000$$

The correlation coefficient of Eq.8 (r^2=0.951) shows that the correlation is significant and the model (Eq.8) has the potential to predict the joint effects for mixture toxicity. The predictive capability of the model and the statistical validity of the modeling are confirmed by application of the 7 other related mixtures to the model (Table 3). The predicted TUs are plotted against the observed ones in Figure 1. It can be seen from Figure 1 that, there is a good consistency between predicted TUs and the observed ones, with r^2=0.953, SE=0.063 and F=100.851 at a level of significance p=0.000. This model therefore appears to be an effective method to predict joint toxic effects. However, before this model can be used confidently, the mechanism of the joint toxic effect has to be revealed by study on the chemical-chemical interactions and toxicodynamic interactions, and therefore all the mixtures applicable to this model can be suggested definitely. Furthermore, this model is not useful for joint effects that result from toxicokinetic interactions. Further study will be carried out by revealment of the joint toxic effects and other approach will be proposed to predict the joint toxic effects that result from toxicokinetic interactions.

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