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Quantitative Structure–Toxicity Relationships for Derivates of Benzanilides to *Daphnia magna*

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Derivates of benzanilides used extensively as intermediates to synthesize pesticides and herbicides are being introduced into the environment. Their environmental behavior and ecological effects should be estimated, however, few published data are available for their toxic effect (Dai, 1998).

During the past two decades the study of quantitative structure-activity relationships (QSARs) has been developed in pharmacology and drug design. Now QSARs are used increasingly to screen and predict toxicity and the fate of chemicals released into the environment. The basic assumption of QSARs is that there is a quantitative connection between the molecular structure of compounds and their biological, chemical, and physical properties. The commonly used methods in QSARs studies are Hansch's method, Linear Solvation Energy Relationship (LSER) method, Molecular Connectivity Indices (MCIs) method, and quantum chemical descriptors. LSER developed by Kamlet and coworkers have successfully applied to many diverse chemical properties (Kamlet 1986; 1987; Blum and Speece 1990). They are based on four molecular characteristics called solvatochromic parameters: V_i, the intrinsic molecular volume; π^*, a measure of polarity or polarizability; and α_m and β_m, measure of the ability to participate in hydrogen bonding as a hydrogen donor or acceptor, respectively. However, the absence of readily available methods to calculate the LSER variables has limited the utility of the model. Hichey and Passino-Reader (1991) developed a method to estimate LSER variables quickly for vast array of possible organic compounds such as those found in the environment, especially the larger compounds of environmental and biological interests. Quantum chemical descriptor is a kind of non-empirical structural descriptor that can be calculated using the quantum chemical software by the chemical structure. It is advantages of quantum structural descriptors that they are not restricted to closely related compounds, they can be easily obtained, and describe clearly defined molecular properties. Because of their advantages, they are widely used in the QSAR studies (Nevalainen 1994; Mekenyan et al. 1994). A variety of test organisms have been

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used to assess environmental toxicity, including bacteria, algae, plankton, zooplankton, fish and so on. Among them, *Daphnia magna*, an important primary consumer and a major food source for vertebrate and invertebrate predators, is a useful test species to study sensitivity to environmental toxicants and has been recognized as a general representative for other freshwater animals (Hebert 1978).

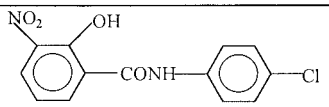
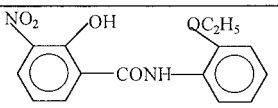
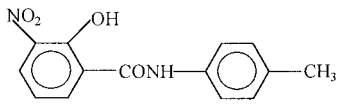
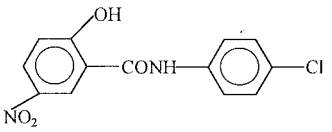
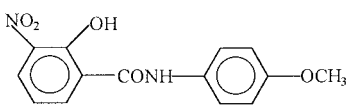
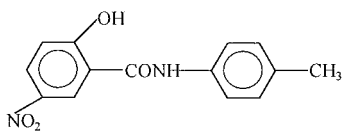
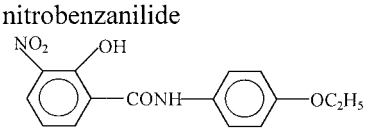
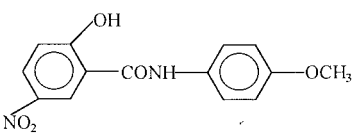
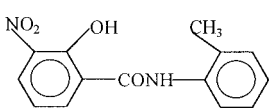
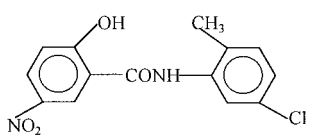
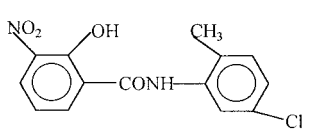
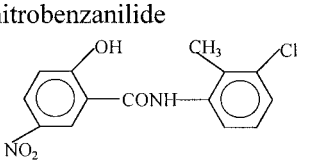
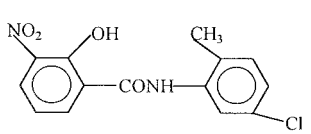
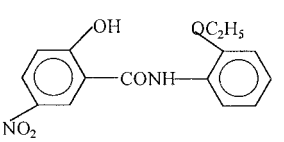
In this paper, toxicities of 14 derivates of benzanilides to *D. magna* were observed, and QSARs correlation using LSER parameters and semi-empirical quantum chemical descriptors were established. The mechanism of toxicity is discussed according to the results. Furthermore, Effectiveness of regression equations in predicting toxicity to *D. magna* for the studied chemicals has been compared. The results should be helpful in evaluating the potential toxicity of these chemicals.

MATERIALS AND METHODS

14 derivates of benzanilides were synthesized in College of Chemistry and Chemical Engineering of Nanjing University. The melting points for 14 derivates of benzanilides measured are same as that reported in the references. Their structures were further confirmed using elemental analysis, GC/MS, IR, and ^{1}HNMR spectra. Their purities were monitored by HPLC to assure purity. Their structures and names for tested chemicals are listed in Table 1.

Dechlorinated tap water (dissolved oxygen concentration>8.0 mg/L; pH=7.4; hardness=99.4 mg/L expressed as $CaCO_3$) was used for maintaining stock cultures and was used as the test medium in all experiments. The *D. magna* used in the experiments were obtained from a culture maintained at Nanjing University. The green algae (*Chlorella pyrenoidoso*) were served as food source for *D. magna*. The acute toxicity tests were based on the static method and nominal concentrations. The concentration range of substituted derivates of benzanilides in which effects were likely to occur was determined from range-finding tests. Three separate assays were performed, involving seven different concentrations of derivates of benzanilides and dilution water controls. Ten neonates (less than 24-hr-old) were placed randomly into each of three replicates test vessels at each exposure level. Because of the short duration of the test, neonates were not fed during the bioassay. All tests were performed at 22 ± 1°C with a 10:14-hr light : dark photoperiod. The number of affected daphnids in each treatment was recorded 48h after the beginning of the test. The effect criterion was immobilization, which was defined as the lack of movement (except for minor movement of the appendages) in response to prodding with a gentle current of water during a 5-s observation period. The results of the three assays were combined and probit analysis was used to calculate the median immobilization concentration of tested chemicals (Table 2).

Table 1. Chemical structures and names of 14 compounds.

No.	structures and names	No.	structures and names
1	 4'-chloro-2-hydroxy-3-nitrobenzanilide	8	 2'-ethoxy-2-hydroxy-3-nitrobenzanilide
2	 4'-methyl-2-hydroxy-3-nitrobenzanilide	9	 4'-chloro-2-hydroxy-5-nitrobenzanilide
3	 4'-methoxy-2-hydroxy-3-nitrobenzanilide	10	 4'-methyl-2-hydroxy-5-nitrobenzanilide
4	 4'-ethoxy-2-hydroxy-3-nitrobenzanilide	11	 4'-methoxy-2-hydroxy-5-nitrobenzanilide
5	 2'-methyl-2-hydroxy-3-nitrobenzanilide	12	 2'-methyl-5'-chloro-2-hydroxy-5-nitrobenzanilide
6	 2'-methyl-5'-chloro-2-hydroxy-3-nitrobenzanilide	13	 2'-methyl-3'-chloro-2-hydroxy-5-nitrobenzanilide
7	 2'-methyl-3'-chloro-2-hydroxy-3-nitrobenzanilide	14	 2'-ethoxy-2-hydroxy-5-nitrobenzanilide

The parameters $V_i/100$, π^*, α_m, β_m of 14 derivates of benzanilides were calculated by the method described by Hickey *et al.* (Table 2). Molecular geometry was optimized and parameters were calculated using the semi-empirical orbital MOPAC6.0 procedures (Stewart 1990) according to methods AM1 (Dewar 1985). MOPAC was used to determine the individual structural parameters influencing toxicity, including the average polarizablity (α), the dipole moment (μ), the energy of the highest occupied molecular orbital (E_{homo}), and the energy of the lowest unoccupied molecular orbital (E_{lumo}), the most positive net atomic charges on hydrogen (qH^+), the largest negative atomic charge on an atom (q^-), the heat of formation (HOF), the total energy (E_{total}), and electronic energy (EE) (Tables 3). The MOPAC was run with the following key words: AM1, ESP, PRECISE, DIPOLE, POLAR, NOINTER. All calculations were run on a personal computer equipped with 32 megabytes of internal memory and supported by Disk Operation System (DOS). The regression analysis was performed using the "Statgraphics" program (STSC, Inc; 1987).

RESULTS AND DISCUSSION

The toxicity data of tested chemicals are given in Table 2. It can be seen from Table 2 that the toxicity of 14 derivates of benzanilides to *D. Magna* varied with their molecular structures. 4'-chloro-2-hydroxy-5-nitrobenzanilide was the most toxic (EC_{50}=–6.330mol/L), whereas 2'-methyl-2-hydroxy-3-nitrobenzanilide was least toxic (EC_{50}=–5.458mol/L). In order to describe the relation between the $logEC_{50}$ of tested chemicals and the LSER parameters, a multiple linear regression analysis was performed. The obtained QSAR equation are presented as follow:

LSER:

$$logEC_{50}=0.36\alpha_m(\pm0.08)+1.67\beta_m(\pm0.20)-3.68\pi^*(\pm0.43)-0.63(\pm0.81)$$
$$n=14,\ R^2=0.905,\ SE=0.10,\ F=42.27,\ p=0.0000 \qquad (1)$$

Where *n* represents the number of samples, *r* is the regression coefficient, *SE* is the standard error of estimates, *F* denotes the *F*-test value, *p* is the significance level of the whole equation. The number in parentheses is the standard error of estimates associated with each coefficient. The F and p values of equation (1) show that the equations are significant. Equation (1) has large correlation coefficient and small SE value. This indicated that $logEC_{50}$ can be predicted by model based on LSER method. The predicted $logEC_{50}$ (using equation (1)) is well fitted with observed $logEC_{50}$ (Fig.1). It can be seen from equation (3) that $logEC_{50}$ can be predicted by model based on quantum chemical method, but, the correlation was not so good with large error for compared with the LSER method. This indicated that LSER methods are more effective than quantum chemical method in predicting $logEC_{50}$ for studied compounds (Table 2).

Table 2. The estimated LSER parameters and the 48-hr $\log EC_{50}$(mol/L) values for 14 tested compounds.

No.	$V_i/100$	π^*	β_m	α_m	$\log EC_{50}$ (mol/L)				
					Obs.	Pre(equ.(1)).	Diff.	Pre(equ.(3)).	Diff.
1	1.44	2.12	1.25	0.42	-6.236	-6.224	-0.012	-5.913	-0.323
2	1.448	1.96	1.31	0.42	-5.568	-5.502	-0.066	-5.662	0.094
3	1.493	2.10	1.51	0.48	-5.644	-5.685	0.041	-5.554	-0.090
4	1.591	2.10	1.51	0.48	-5.796	-5.626	-0.170	-5.692	-0.104
5	1.448	1.96	1.30	0.42	-5.458	-5.517	0.059	-5.631	0.173
6	1.538	2.08	1.26	0.42	-5.931	-5.993	0.062	-5.977	0.046
7	1.538	2.08	1.26	0.42	-5.973	-5.993	0.020	-6.070	0.097
8	1.591	2.10	1.51	0.48	-5.558	-5.626	0.068	-5.650	0.092
9	1.44	2.22	1.28	1.20	-6.330	-6.261	-0.069	-6.334	0.004
10	1.448	2.06	1.34	1.20	-5.558	-5.539	-0.019	-5.523	-0.035
11	1.493	2.20	1.54	1.26	-5.655	-5.722	0.067	-5.622	-0.033
12	1.538	2.18	1.29	1.20	-6.009	-6.030	0.021	-5.917	-0.092
13	1.538	2.18	1.29	1.20	-6.027	-6.030	0.003	-6.098	0.071
14	1.591	2.20	1.54	1.26	-5.665	-5.663	-0.002	-5.763	0.098

Table 3. Molecular structure descriptors derived from optimized geometric structures of the studied compounds using AM1 method.

No.	α	μ	$-E_{homo}$	$-E_{lumo}$	qH^+	q^-	HOF	$TE\times10^3$	$EE\times10^4$
	[a.u.]	[a.u.]	[eV]	[eV]	[a.c.u.]	[a.c.u.]	[kcal/mol]	[eV]	[eV]
1	152.798	1.741	9.340	1.467	0.274	0.392	-23.543	3.854	2.275
2	154.262	2.096	9.175	1.374	0.272	0.393	-24.480	3.649	2.269
3	160.343	2.468	9.007	1.353	0.273	0.393	-54.343	3.969	2.475
4	169.617	2.513	8.985	1.342	0.272	0.393	-59.998	4.125	2.652
5	152.800	1.518	9.156	1.384	0.273	0.394	-23.967	3.649	2.304
6	159.937	1.283	9.274	1.416	0.274	0.394	-30.740	4.010	2.519
7	160.603	1.534	9.344	1.454	0.274	0.392	-28.889	4.009	2.508
8	167.705	3.287	9.082	1.093	0.269	0.375	16.347	4.122	2.709
9	163.178	3.188	8.550	2.445	0.265	0.359	56.471	3.850	2.176
10	154.160	2.211	9.177	1.228	0.247	0.361	-23.781	3.649	2.255
11	162.910	2.937	8.832	2.213	0.243	0.358	30.636	3.966	2.469
12	161.628	2.835	9.308	1.277	0.247	0.359	-30.003	4.009	2.490
13	163.960	3.089	9.209	2.304	0.245	0.350	56.079	4.006	2.474
14	171.560	3.173	8.914	1.200	0.256	0.361	-58.841	4.125	2.676

The student *t* values for partial correlation coefficient are 4.36, 8.36, and –8.93,

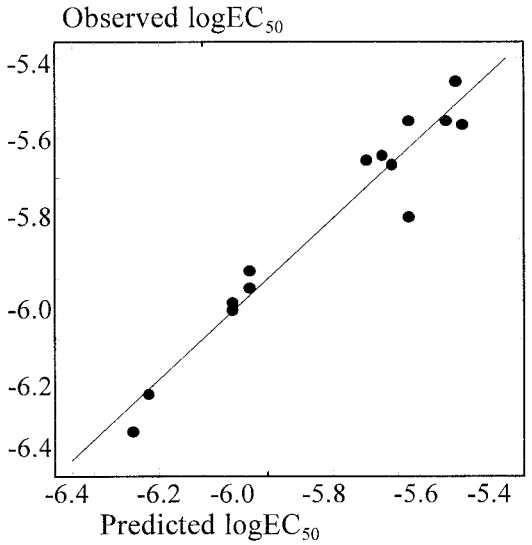


Figure 1. Plot of observed $logEC_{50}$ vs. calculated $logEC_{50}$ using equation (1)

for α_m, β_m, and π^*, respectively. This indicated that π^* descriptor is the most significant factor for $logEC_{50}$. The π^* descriptor is a measure of molecular dipolarity or polarizability. As may be concluded from the magnitude of partial correlation coefficients that α_m and β_m terms are less important for $logEC_{50}$ compared with the π^* term. In the equation (1), the $V_i/100$ terms is not included because they are not significant at the 95% confidence level, and this is in accordance with other studies (Yang, 1997). The parameter α_m and β_m have a positive sign, which means that the larger α_m and β_m, the larger is the $logEC_{50}$ value, the less toxicity to *D. Magna*. The parameter π^* has a negative sign, which means that the larger π^*, the smaller is the $logEC_{50}$ value. This indicates that increasing molecular dipolarity or polarizability will increase the toxicity to *D. Magna* for the studied compounds.

Lots of studies have shown that hydrophobicity of a compound is the basic property that determines the bioactivity of that compound. Many researchers recommended the use of octanol/water partition coefficient as hydrophobicity descriptors in QSAR studies. Here we use Kow as hydrophobicity descriptor in the following QSAR studies. Kow values of 14 tested chemicals were determined by shake-flask method (Dai *et al.* 1998). We obtained the following relationship by simple linear regression:

$$logEC_{50} = -4.286(\pm 0.502) - 0.692 logKow(\pm 0.226)$$
$$n=14,\ R=-0.66,\ SE=0.212,\quad p=0.0098 \qquad (2)$$

Equation (2) indicated that hydrophobicity is the dominant factor determining the toxicity of these studied compounds to *D. magna,* and the equation show that the toxicity increases with increasing the hydrophobicity of the compounds. However, the correlation coefficients of equation (2) is not very high and the standard error is relatively large, which shows that besides hydrophobic interactions, other interactions may play important roles in determining the toxicity.

It has been suggested that an adequate model should include as many descriptors as possible to increase the probability of good characterization of compounds. The quantum chemical parameters which have clear "chemical sense" are used in the analysis of toxicity. Therefore, 9 MOPAC derived descriptors were used in this study. By stepwise regression analysis, the following high-quality regression equations were obtained:

AM1:

$$\log EC_{50}=0.56(\pm1.35)-0.97\log Kow(\pm0.21)-0.18E_{lumo}(\pm0.11)-15.03qH^{+}(\pm4.02)$$
$$n=14,\ R^2=0.7841,\ SE=0.14,\ F=12.11,\ p=0.0011 \qquad (3)$$

The F and p values of equation (3) show that the equations are significant. Equation (3) has large correlation coefficients and small SE values. Comparing equation (2) with equation (3), we find that the correlation coefficient is significantly improved and the standard error of estimation is greatly reduced by introducing quantum chemical parameters into equations. It can be seen from equation (3) that $\log EC_{50}$ can be predicted by model based on quantum chemical method, though, the regression coefficient and standard error of estimates of equation (3) were not so good for compared with equation (1). The number of independent variables in equation (3) was the same as in equation (4), and the descriptors in equation (3) have definite physio-chemical sense. It may be concluded that AM1 method is effective in predicting toxicity for studied compounds. The calculated and residual values of $\log EC_{50}$ based on equation (3) are listed in Table 2. It can be seen from Table 2 that the experimental values of $\log EC_{50}$ were close to the predicted. The average errors of estimated log EC_{50} was 0.10 log units.

The cross correlation of descriptors in equation (3) is examined by the variance inflation factor (VIF). The VIF is defined as $1/(1-r^2)$, where r is the correlation coefficient of the subject independent variable against all other independent variables in the regression. A value of 1.0 indicates no correlation, with values under 5.0 being acceptable. Values over 10.0 indicate an unstable regression that should be reexamined (Famini 1997). The correlation coefficients of the independent variables and the VIF values for equation (3) were given in Table 4.

The result indicates that the independent variables in equation (3) did not exist cross correlation. The obtained equation is of significance.

Table 4. Correlation coefficient matrix for significant independent variables and the variance inflation factors (VIF) for equation (3).

	E_{lumo}	qH^+	logKow	R^2(adj.)	VIF
E_{lumo}	1.0000			0.1179	1.13
qH^+	0.0901	1.0000		0.2477	1.33
logKow	-0.3771	0.5181	1.0000	0.3494	1.54

By stepwise selection, the energy of the lowest unoccupied molecular orbital (E_{lumo}), and the most positive net atomic charges on hydrogen (qH^+), were selected into the equation (3). Furthermore, the student *t* values for partial correlation coefficient are - 4.68, -1.75 and 3.74, for logKow, E_{lumo} and qH^+ descriptors in equation (3), respectively. This indicated that logKow descriptor is the most significant factor for $logEC_{50}$. In addition, qH^+ descriptor is related the ability of the solutes to participate in hydrogen-bonding interactions with membrane molecules of the tested organisms. As may be concluded from the magnitude of partial correlation coefficients, the kind of interactions is rather less important for the toxicities compared with the hydrophobicity interaction. The last principal descriptor E_{lumo} determines the toxicity to *D. Magna* for test compounds. Its participation in the correlation equation is even less than qH^+ term, indicating that it is not important for toxicity. E_{lumo} descriptors are related the ability of the solutes to participate in electron pair donor-acceptor interactions with membrane molecules of the tested organisms. The parameters logKow, E_{lumo} and qH^+ have a negative sign, which means that the large logKow, E_{lumo} and qH^+, the smaller is the $logEC_{50}$ value, the larger toxicity to *D. Magna*.

In summary, the information presented in this study shows that these models based on LSER method and quantum chemistry descriptors can be used to predict $logEC_{50}$ value. Between them, the LSER method is more effective method in predicting $logEC_{50}$ value for studied compounds. The E_8 descriptor seems to be dominant in retention mechanism. These models can provide a useful starting point for predicting the potential environmental contaminant of structure-related compounds.

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