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Toxicity Prediction of Antibiotics on Luminescent Bacteria, *Photobacterium phosphoreum*, Based on Their Quantitative Structure–Activity Relationship Models

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Abstract Acute toxicity ($EC_{50-30\ min}$) and chronic toxicity ($EC_{50-24\ h}$) of 21 antibiotics on *Photobacterium phosphoreum* was observed and Quantitative structure–activity relationship (QSAR) models were developed, respectively. By comparing these two QSAR models, the following model was established, $\log(1/EC_{50-24\ h}) = 1.8283 + 1.1503\log(1/EC_{50-30\ min}) + 0.2872\text{Elomo} - 0.0901\text{DM} + 0.0003\text{PMIZ} + 0.0088\text{PSA} - 0.0382\text{SD}$, $r^2 = 0.8513$, where Elomo is lowest occupied molecular energy, DM is dipole moment, PMIZ is principal moment of inertia Z, PSA is polar surface area, and SD is sum of degrees. It provides a good way for us to obtain $EC_{50-24\ h}$ from $EC_{50-30\ min}$, because the later is far easier to observe than the former.

Keywords Toxicity · QSAR · Antibiotics · *Photobacterium phosphoreum*

Antibiotics are widely used as human and veterinary medicines and animal feed additives. Thirty to ninety percent of antibiotics are excreted into urine or feces, and then got into the environment via many pathways such as domestic treatment system effluents and livestock wastewater because of ineffective removal and improper disposal (Brown et al. 2006). A great variety of antibiotics have been detected in surface water and even in drinking water up to now (Benotti et al. 2009). Consequently, toxicity data of antibiotics is greatly needed for the understanding of their ecological impacts and the performance of environmental risk assessments. Studies about the toxicity effects of antibiotics have been performed with aquatic organisms in recent years, including luminescent bacteria, algae, invertebrates and fish (Froehner et al. 2000; Robinson et al. 2005; Park and Choi 2008). Most of these studies focus on acute toxic effects, and there are few data on chronic toxicity. However, chronic toxicity data is more creditable for environmental risk assessments, because it reflects the low concentration (ng L^{-1} to low μg L^{-1}) and long-term exposure of antibiotics on organisms in the actual environment.

There are two ways for us to obtain the chronic toxicity data. One is that chronic toxicity data can be obtained by tests, but the chronic tests are hindered by the long test period and operating difficulties in testing. The other is that chronic toxicity data can be obtained by extrapolating from the acute toxicity data because acute toxicity data are easy to be observed. The extrapolation approach is that an assessment factor of 10 is applied to account for the uncertainty from acute toxicity to chronic effects in the environmental risk assessments. However, this approach has been doubted because the assessment factor of 10 is too rough to eliminate the difference between acute and chronic toxicity (Carlsson et al. 2006; Daughton and Ternes 1999). Fortunately, the quantitative structure–activity relationship (QSAR) has proven to be a precise approach to study chemical toxicity by correlating effects with various properties for many years (Yuan et al. 1997). Therefore, studying the QSARs may lead us to understand

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the relationship between acute and chronic toxicity, and then may provide a way for us to extrapolate chronic toxicity data from acute toxicity data. The objectives of this study were: (1) to observe the acute and chronic toxicity of 21 widely used antibiotics on *Photobacterium phosphoreum* (*P. phosphoreum*); (2) to develop and compare their acute and chronic QSAR models; and (3) to establish an approach to predict chronic toxicity from acute toxicity by introducing physicochemical descriptors obtained from QSAR models.

Materials and Methods

Twenty-one antibiotics, including six fluoroquinolones, three tetracyclines, three chloramphenicols, seven sulfonamides, trimethoprim and ciprofloxacin were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). They were 95.0–99.5% pure.

The freeze-dried power of *P. phosphoreum* was purchased from the Institute of Soil Science, Academic Sciences, Nanjing, China. Bacteria were inoculated on an agar plate and further incubated in liquid culture medium at 21 ± 0.5°C for 16–20 h to reach the logarithmic growth phase for acute toxicity tests. The culture medium consists of 0.5% yeast extract, 0.5% tryptone, 0.3% glycerol, 3% NaCl, 0.5% Na_2HPO_4, and 0.1% KH_2PO_4, and is adjusted to pH 7.0 ± 0.5. The preparation of bacterial suspension for the chronic tests was performed by using a small inoculum and concentrated medium. The bacteria were precultured to the logarithmic stage and used as the inoculum. The inoculum was diluted with 2× concentrated medium at the ratio of 1:5 to supply enough nutrition for the growth of bacteria during the long incubation term, the final bioluminescence of the bacterial suspension was approximately one tenth of the inoculum bioluminescence. The salinity of the test system was adjusted to 3% with NaCl solution to keep appropriate osmotic pressure for the metabolism of *P. phosphoreum*. For the stability of bacteria growth, microplates were incubated in the horizontal shaker at 125 rpm min^{-1} at 21 ± 0.5°C during the test period (Gellert et al. 1999).

Both the acute and chronic toxicity tests of each antibiotic were performed in 96-well microplates, in accordance with microplate toxicity analyses (Zhang et al. 2008). The test system (200 μL for each well) contained 100 μL of aqueous dilution series of test antibiotics and 100 μL of working bacterial suspension. For each antibiotic, a series of 12 concentrations was used for the concentration–response curves (CRCs) by an appropriate dilution factor. Microplates were incubated for 30 min and 24 h exposures for acute and chronic toxicity, respectively. The luminescence of the bacteria was determined using a SpectraMax M5 (Molecular Devices Inc., USA). The toxicity was expressed as inhibition of bioluminescence. All toxicity tests were performed in triplicate for each antibiotic. The two-parameter formula *Weibull* or *Logit* was used to fit the CRCs, and the median effective concentration (EC_{50}) was derived.

Twenty-one frequently used physicochemical descriptors (including two hydrophobic, five electronic, two thermodynamic, and 12 steric property descriptors) were considered for the QSAR development (Table 1). They were calculated by Chemoffice 2005 version 9.0. To select the relevant descriptors from them, stepwise multiple linear regressions were performed for the observed toxicity data ($\log(1/EC_{50})$) against all 21 descriptors using SPSS 16.0 software. According to the regression results, QSAR models were developed with the relevant descriptors. The statistical quality of QSAR models was evaluated by the square of the correlation coefficient (r^2), root mean standard error (RMSE), Fischer ratio (F), and the significant level (p). The stability and prediction ability of models were examined by using leave-one-out (LOO) cross-validation, with the cross-validated squared correlation coefficient (q^2) and the root mean standard error from LOO cross-validation (RMSV).

Results and Discussion

Acute toxicity values of antibiotics on *P. phosphoreum* after 30 min of exposure, expressed as $\log(1/EC_{50\text{–}30\ min})$, are listed in Table 2. The $EC_{50\text{–}30\ min}$ values ranged from 45.68 mg L^{-1} (ciprofloxacin, $\log(1/EC_{50\text{–}30\ min}) = 3.91$ in Table 2) to 1,225.01 mg L^{-1} (trimethoprim, $\log(1/EC_{50\text{–}30\ min}) = 2.38$ in Table 2). Acute $EC_{50\text{–}30\ min}$ can not be determined for sulfadiazine, sulfamethazine, sulfamethoxydiazine, and cephalexin, because they only generated inhibition effects of 13.65%, 10.06%, 21.47%, and 10.82% at their corresponding water solubility, respectively.

Furthermore, chronic toxicity data ($EC_{50\text{–}24\ h}$) was determined after 24 h exposure and the results are listed in Table 2. It can be seen from Table 2 that, ciprofloxacin was found to be the most toxic compound with the $EC_{50\text{–}24\ h}$ value of 0.0109 mg L^{-1} ($\log(1/EC_{50\text{–}24\ h}) = 7.53$ in Table 2), followed by other five fluoroquinolones (enrofloxacin, norfloxacin, fleroxacin, sarafloxacin and ofloxacin). The result is similar to the study of Park and Choi (2008) in which enrofloxacin was observed to be the most toxic on *Daphnia magna* and *Moina macrocopa* among four different classes of antibiotics. Meanwhile, sulfapyridine exhibited the lowest toxicity among all test antibiotics, with the $EC_{50\text{–}24\ h}$ value of 22.80 mg L^{-1} ($\log(1/EC_{50\text{–}24\ h}) = 4.04$ in Table 2). $EC_{50\text{–}24\ h}$ values of antibiotics in this

Table 1 Physicochemical descriptors used in this study

Type	Descriptor	Description
Hydrophobic	Kow	Octanol/water partition coefficient
	Dow	Derived from Kow and adjusted for the pH of reaction medium and p*Ka* of the compound
Electronic	Ehomo	Highest occupied molecular energy
	Elomo	Lowest occupied molecular energy
	DM	Dipole moment
	EE	Electronic energy
	Te	Total energy
Thermodynamic	Hf	Heat of formation
	MR	Molar refractivity
Steric	Mass	Exact mass
	PSA	Polar surface area
	PMIX, Y and Z	Principal moment of inertia X, inertia Y and inertia Z
	Balaban	Balaban index
	ClsC	Cluster count
	Diam	Diameter
	Shape	Shape index
	WIndx	Weiner index
	SD	Sum of degrees
	TIndx	Molecular topological index

study are in the same level as those in previous study (Backhaus and Grimme 1999; Backhaus et al. 2000).

Comparison of $EC_{50–30\ min}$ and $EC_{50–24\ h}$ showed that $EC_{50–24\ h}$ values are two to three orders of magnitude lower than $EC_{50–30\ min}$ ones. The result indicates that, toxicity could be caused after a long-term exposure, even if there is no effect after a short-time exposure. The similar result was reported that sulfathiazole did not exhibit highly toxic effect on *D. magna* at 24 h ($EC_{50–24\ h}$, 616.7 mg L^{-1}), but after 48 h of exposure, the toxicity on *D. magna* increased by four times ($EC_{50\text{-}48h}$, 149.3 mg L^{-1}) (Park and Choi 2008). Therefore, the long-term toxicity data should be used instead of the short-term data in risk assessments of antibiotics.

Based on these observed toxicity data, QSAR models were developed for $\log(1/EC_{50–30\ min})$ and $\log(1/EC_{50–24\ h})$ against 21 physicochemical descriptors by stepwise multiple linear regression, respectively. Five descriptors were entered into the QSAR models, their values are shown in Table 2. Acute and chronic QSAR models were expressed as Eqs. 1 and 2, respectively.

$$\log(1/EC_{50–30\ min}) = 1.3637 - 0.0314DM + 0.0482SD - 0.0081PSA - 0.3452Elomo$$
$$n = 17,\ r^2 = 0.9339,\ r = 0.9664,\ RMSE = 0.1401,$$
$$F = 42.401,\ p = 0.0001\,(Estimation)$$
$$n = 17,\ q^2 = 0.8144,\ q = 0.9024,\ RMSV = 0.2379\ (LOO) \quad (1)$$

$$\log(1/EC_{50–24h}) = 3.4471 + 0.00049PMIZ - 0.1270DM$$
$$n = 21,\ r^2 = 0.7979,\ r = 0.8933,\ RMSE = 0.5227,$$
$$F = 35.533,\ p = 0.0001\,(Estimation)$$
$$n = 21,\ q^2 = 0.7395,\ q = 0.8599,\ RMSV = 0.5956\ (LOO) \quad (2)$$

It can be seen from Eq. 1 that, a four-descriptor QSAR model was developed for the acute toxicity data, with both good correlation relationship ($r^2 = 0.9339$ and RMSE = 0.1402) and high stability and prediction ($q^2 = 0.8144$ and RMSV = 0.2379). As shown in Eq. 1, the acute toxicity data of antibiotics have negative correlations with dipole moment (DM), lowest occupied molecular energy (Elomo), and polar surface area (PSA), and positive correlations with sum of degrees (SD). Lowest occupied molecular energy and dipole moment are electronic descriptors and relate to the relative activity of compounds. Chemicals with lower lowest occupied molecular energy are more likely to accept electronic, easier interact with other molecules at a higher speed, and therefore potentially result in greater toxicity. Polar surface area and sum of degrees represent the steric information of compounds; polar surface area is the descriptor showing the correlation with passive molecular transport through membrane. The acute QSAR model demonstrates that the acute toxicity of antibiotics is mainly related with their electronic and steric properties, and it can be described by dipole moment, lowest occupied molecular energy, polar surface area, and sum of degrees.

Table 2 Toxicity results of the test antibiotics and physicochemical descriptions entered into QSAR models

Compound	MW[a]	log(1/$EC_{50-30\ min}$) (mol L^{-1})[b]	log(1/$EC_{50-24\ h}$) (mol L^{-1})[b]	Elomo (ev)[c]	DM[d]	PMIZ[e]	PSA[f]	SD[g]
Ciprofloxacin	368	3.91 (3.84–3.99)	7.53 (7.40–7.70)	−0.532	−5.649	4,917.6	86.6	54
Norfloxacin	319	3.50 (3.45–3.54)	7.21 (7.40–7.70)	−0.527	−4.722	4,621.0	86.6	50
Ofloxacin	361	3.81 (3.73–3.92)	6.73 (6.63–6.85)	−0.619	−7.986	5,308.9	90.6	58
Fleroxacin	369	3.80 (3.74–3.87)	6.91 (6.81–7.04)	−0.748	−6.082	6,004.1	76.5	56
Sarafloxacin	422	3.91 (3.89–4.02)	6.84 (6.65–7.42)	−0.844	−1.791	6,447.5	86.6	62
Enrofloxacin	359	3.90 (3.81–4.00)	7.24 (7.15–7.35)	−0.490	−6.348	6,536.2	76.5	58
Enoxacin	320	3.85 (3.63–3.93)	6.81 (6.63–7.10)	−0.600	−3.620	4,618.5	97.9	50
Tetracycline	445	3.23 (3.16–3.24)	6.60 (6.33–6.69)	−0.721	4.326	6,767.9	201.4	72
Oxytetracycline	461	3.01 (2.96–3.01)	6.82 (6.63–7.04)	−0.899	3.004	6,893.2	255.6	74
Doxycycline	445	3.84 (3.67–3.92)	6.58 (6.37–6.74)	−0.822	−3.603	6,621.8	201.4	72
Chlortetracycline	515	3.72 (3.57–3.94)	6.71 (6.53–7.02)	−0.998	−5.192	6,784.5	201.4	74
Chloramphenicol	323	2.87 (2.83–2.91)	5.97 (5.85–6.14)	−1.566	2.453	4,020.1	130.0	40
Florfenicol	358	3.11 (3.07–3.16)	6.21 (6.13–6.32)	−1.456	−3.831	3,825.1	103.5	42
Thiamphenicol	356	2.77 (2.73–2.80)	5.80 (5.66–6.00)	−1.419	−3.237	4,597.7	127.3	42
Sulfamethoxazole	258	2.67 (2.63–2.73)	4.42 (4.32–4.56)	−1.379	3.519	2,100.7	105.7	36
Sulfadiazine	250	–[h]	4.24 (4.12–4.41)	−1.149	3.747	2,773.9	102.9	36
Sulfamethazine	278	–	4.16 (3.32–4.56)	−1.152	−0.856	3,745.8	102.9	40
Sulfamerazine	264	2.51 (2.46–2.56)	4.31 (4.14–4.61)	−1.112	4.094	3,195.8	102.9	38
Sulfamethoxydiazine	280	–	4.41 (4.12–4.41)	−1.108	4.461	4,068.3	117.0	40
Sulfadimethoxine	310	2.50 (2.42–2.60)	4.43 (4.26–4.72)	−1.284	4.256	4,488.8	119.8	44
Sulfapyridine	249	2.74 (2.69–2.80)	4.04 (3.88–4.28)	−0.990	4.950	2,785.7	91.4	36
Sulfachlororyridazine	285	2.63 (2.58–2.69)	4.80 (4.71–4.90)	−1.511	2.801	4,215.6	102.9	38
Sulfamethoxypyridazine	280	2.49 (2.44–2.54)	4.36 (4.21–4.58)	−1.080	7.061	4,056.1	117.0	40
Sulfachinoxalin	330	2.55 (2.49–2.61)	4.53 (4.42–4.69)	−1.227	0.442	4,776.4	102.9	46
Trimethoprim	290	2.38 (2.29–2.48)	5.08 (4.91–5.34)	0.191	−0.080	3,624.6	110.7	44
Cephalexin	347	–	5.70 (5.53–5.95)	−0.473	1.210	4,302.5	129.1	52

[a] MW is molecular weight

[b] Mean log(1/EC_{50}) with 95% confidence

[c] Elomo is lowest occupied molecular energy

[d] DM is dipole moment

[e] PMIZ is principal moment of inertia Z

[f] PSA is polar surface area

[g] SD is sum of degrees

[h] Acute EC_{50} was not calculated at the corresponding water solubility of the compound

In the same way, a two-descriptor chronic QSAR model was developed as Eq. 2. The relationship was characterized by $r^2 = 0.7979$ and RMSE = 0.5227, and $q^2 = 0.7395$ and RMSV = 0.8599. The chronic QSAR model shows that principal moment of inertia Z (PMIZ) and dipole moment are the most important descriptors for the chronic toxicity of antibiotics among the 21 descriptors, and indicates that electronic and steric properties play important roles in the chronic toxicity of antibiotics.

Comparison of the acute QSAR model and the chronic one shows that, the toxicity of antibiotics is well correlated to dipole moment, lowest occupied molecular energy, polar surface area, sum of degrees, and principal moment of inertia Z. Therefore, by introducing these five descriptors, an approach between log(1/$EC_{50-24\ h}$) and log(1/$EC_{50-30\ min}$) was established as follows,

$$\begin{aligned}\log(1/EC_{50-24\,h}) &= 1.8283 + 1.1503\log(1/EC_{50-30\,min}) \\ &\quad + 0.2872\mathrm{Elomo} - 0.0901\mathrm{DM} \\ &\quad + 0.0003\mathrm{PMIZ} + 0.0088\mathrm{PSA} - 0.0382\mathrm{SD} \\ &\mathrm{n} = 17,\ r^2 = 0.8513,\ r = 0.9227, \\ &\mathrm{RMSE} = 0.4196,\ F = 9.541,\ p = 0.0001(\mathrm{Estimation}) \\ &\mathrm{n} = 17,\ q^2 = 0.6173,\ q = 0.7857, \\ &\mathrm{RMSV} = 0.7087\ (\mathrm{LOO}) \end{aligned} \quad (3)$$

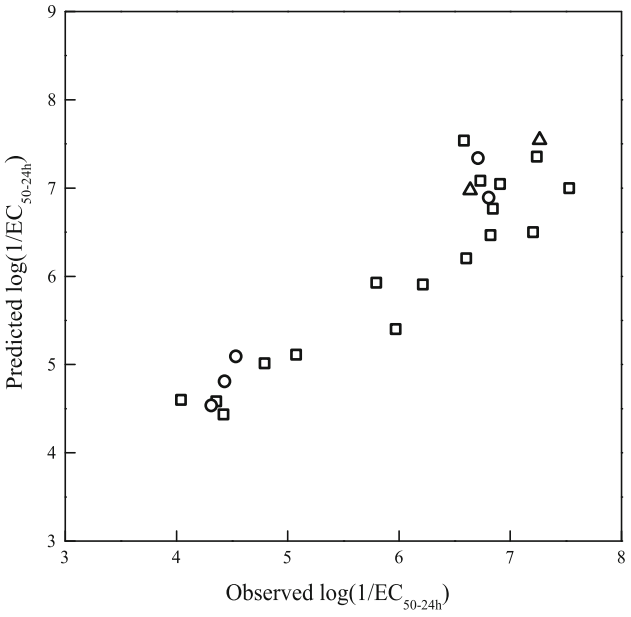

Fig. 1 Plot of the observed $\log(1/EC_{50-24\ h})$ and the predicted values according to Eq. 3. (*Open square*) data involved in the model, (*Open circle*) data used for validation, (*Open triangle*) data reported in the literatures

Eq. 3 has a good correlation relationship ($r^2 = 0.8513$, RMSE = 0.4196) and stability and prediction ($q^2 = 0.6173$ and RMSV = 0.7087). The equation shows that $EC_{50-24\ h}$ values can be well predicted from the corresponding $EC_{50-30\ min}$ values with the combination of chemical properties.

The prediction ability of Eq. 3 and the statistical validity of the modeling are confirmed by application of the five other related chemicals to the model. The predicted $\log(1/EC_{50-24\ h})$ is plotted against the observed ones in Fig. 1. There is a good agreement between the predicted and observed values with $r^2 = 0.9631$ at a level of significance $p < 0.0001$. The residuals between them range from 0.09 to 0.63. Since the validation data were not involved in any process of QSAR model development, the generally satisfactory agreement between the observed and predicted values therefore demonstrates that the model can be used to predict chronic toxicity of antibiotics. Moreover, the good consistency between the predicted $\log(1/EC_{50-24\ h})$ of antibiotics involved in QSAR development and their corresponding observed values is also showed in Fig. 1.

Furthermore, the prediction ability of Eq. 3 was verified by using toxicity data of antibiotics reported in the literatures. Toxicity of tetracycline was reported as $EC_{50-30\ min}$ of 19.6 mg L^{-1} and $EC_{50-24\ h}$ of 0.0241 mg L^{-1} on *V. fischeri* (Backhaus et al. 1997); toxicity of chloramphenicol was also reported as $EC_{50-30\ min}$ of 19.84 mg L^{-1} and $EC_{50-24\ h}$ of 0.074 mg L^{-1} on *V. fischeri* (Froehner et al. 2000). The predicted $\log(1/EC_{50-24\ h})$ was calculated for them based on the corresponding reported $EC_{50-30\ min}$ according to Eq. 3. Residuals between the predicted and reported $\log(1/EC_{50-24\ h})$ were 0.3 for both of the examples (Fig. 1). The results further indicate a good prediction ability of Eq. 3, even for different luminescent bacteria species and in different test conditions.

In addition, this model can be applied in the risk assessments of antibiotics. For example, risk quotients (RQs) of ciprofloxacin and trimethoprim were calculated to be 1.82 and 0.01 (Table 3), and this indicates the unacceptable risk of ciprofloxacin and the acceptable exposure of trimethoprim. These results of risk assessments are the same as that in the literature (Halling-Sørensen et al. 2000). Therefore, the risk assessments of antibiotics, based on the toxicity data predicted by the model in this study, can mirror their relative toxicities to the real world.

Table 3 Environmental risk assessments based on the predicted chronic toxicity data from the model in this study[a]

Compound	Predicted $EC_{50-24\ h}$ (μg L^{-1})[b]	AF	PEC (μg L^{-1})[c]	Risk assessments	
				In this study	In the reference[c]
Ciprofloxacin	36.83	100	0.67	Unacceptable (RQ = 1.82)	Unacceptable (RQ = 13.3)
Trimethoprim	2,245	100	0.17	Acceptable (RQ = 0.01)	Acceptable (RQ = 9.5×10^{-4})

[a] Environmental risk is characterized by the Risk Quotient. If the RQ ≥ 1, the ecological risk is considered to be unacceptable; if the RQ < 1, the ecological risk is acceptable. The RQ is calculated as follow: RQ = PEC × AF/EC_{50}. PEC is the predicted environmental concentration of the compound. AF is assessment factor which is applied to eliminate differences between the toxicity in the laboratory and real environment. Based on the EU draft guidelines, AF is 100 if chronic toxicity EC_{50} is available

[b] The chronic toxicity data is predicted by using Eq. 3

[c] Halling-Sørensen et al. (2000)

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