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Acute Toxicity Test with *Daphnia magna*: An Alternative to Mammals in the Prescreening of Chemical Toxicity?

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In this study, the association between the acute toxicity of 15 compounds to *Daphnia magna*, expressed as 24- and 48-h LC_{50} values, and the corresponding oral LD_{50} values for the rat was tested. Since there was evidence of a strong relationship between the two species, the sample was extended to 54 cases by including the values for acute toxicity to *D. magna* and rat of more chemicals published by other authors. Thus, a total of 54 data points were further used to ascertain the relationship between the acute toxicity of chemical compounds to *D. magna* and that to the rat. To summarize its validity, the *D. magna* test is more specific than sensitive as an indicator of toxicity to the rat. When it is used with a chemical that has a high probability of being very toxic to *D. magna* (LC_{50}< 0.22 mg/L), the test provides considerable information if it is positive, virtually giving evidence of toxicity to the rat (with a probability of 0.83). On the other hand, a negative test (*D. magna* LC_{50}> 0.22 mg/L) has a probability of correctly assigning nontoxicity to the rat equal to 0.74. This study and results published by other authors provide good evidence of the applicability of using invertebrate tests as prescreening methods, thus considerably reducing the number of mammals required in toxicity testing.

***Key Words: Daphnia magna*; mammals; rat; acute toxicity; prescreening method.**

INTRODUCTION

In 1959, Russell and Burch in their book, The Principles of Humane Experimental Technique, formally proposed the "3 R's" concept: reduction, refinement, and replacement. Since then, alternative methods to experimentation with highly sensitive animals, particularly mammals, have been developed and some have been introduced in toxicology. The recognition that a precise LD_{50} value for mammals is not required for notification of new chemicals leads to the development of alternative methods to the traditional LD_{50} test for acute oral toxicity determination. Some of these methods (fixed dose procedure, acute toxic class method, and up-and-down procedure) have now been accepted for regulatory purposes and must be used preferentially to the LD_{50} test (Tichias *et al*., 1998).

Studies published in the last decades suggest that acute tests with invertebrates may be used as first screening methods for the assessment of the lethal toxicity of new chemicals to mammals and humans. For example, Neuhauser and collaborators (1985a, b) proposed a rank classification of substances based on acute toxicity (LC_{50} values) to the earthworm *Eisenia foetida* which corresponds to the actual classification based on rat LD_{50} values, while Khangarot and co-workers (Khangarot and Ray, 1988; Khangarot *et al*., 1987) found a high correlation between the acute toxicity of some metals to the crustacean cladoceran *Daphnia magna* and the corresponding LD_{50} values for the mouse and rat. Furthermore, a study published some years ago by Calleja and Persoone (1992) reports the predictive screening potential of some aquatic invertebrate tests for acute oral toxicity in humans better than the rat LD_{50} test for some chemicals. The major advantage of using invertebrate bioassays as prescreening methods is reduction of the number of mammals required for toxicity testing. Since these methods are *in vivo* tests, the biotransformation of chemicals is taken into account and, from this point of view, they seem to be preferable to *in vitro* methods that have been considered to evaluate human acute toxicity (Ekwall *et al*., 1989, 1998) for some purposes. In addition, they are less expensive than bioassays with mammals and require less space. The main difficulty in the use of invertebrate tests as prescreening tests is the difference of biological organization level relative to mammals. Although this difference should be considered, it should be remembered that the objective is using them as prescreening methods. Therefore, a final bioassay with a small number of mammals should be carried out. Despite this requirement, the routine use of

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invertebrate tests will represent a significant reduction in the number of mammals used for regulatory purposes.

In this study, the association between the acute toxicity of 15 compounds to *Daphnia magna*, expressed as 24- and 48-h LC_{50} values, and the corresponding oral LD_{50} values to the rat was investigated. Since there was evidence of a strong relationship between the two species, the sample was expanded to 54 cases by including values for acute toxicity to *D. magna* and rat of more chemicals published by other authors. Thus, a total of 54 data points were further used to ascertain the relationship between acute toxicity of chemical compounds to *D. magna* and the rat.

MATERIAL AND METHODS

Parent animals. Parent animals were individually cultured in 100 mL of ASTM hard water (ASTM, 1980) with an organic additive (Baird *et al.*, 1989b) and fed *Chlorella vulgaris* (0.322 mg carbon/day). The photoperiod was 16 h L:8 h D and the temperature was $20 \pm 1^{\circ}C$.

Acute Daphnia toxicity tests. All experiments were carried out with animals from clone A (Baird *et al.*, 1989a) and initiated with third to fifth brood neonates (< 24 h old). Test medium was ASTM hard water (ASTM, 1980) and the animals were not fed during the tests. Twenty animals per treatment in groups of five per 100 mL of test solution were used. Temperature and photoperiod were as described above. Oxygen concentrations and pH levels were determined 0, 24, and 48 h after the start of the test. The measured effect was death recognized as immobilization for 15 s after stimulation by a bright light.

Test solutions. Tested substances were paraoxon, parathion, chlorpyrifos, 3, 4-dichloroaniline (DCA), cadmium choride, mercurous chloride, copper sulfate, zinc sulfate, chromous chloride, sodium dichromate, potassium dichromate, sodium bromide, sodium dodecyl sulfate (SDS), dodecyl benzyl sulfonate (DBS), ethanol, and methanol. For each chemical, test concentrations were prepared by dilution with ASTM hard water of a stock solution in nanopure water (conductivity 5 μS/cm), except for parathion for which the stock solution was prepared in ethanol. Actual concentrations of DCA, parathion, and chlorpyrifos, considered difficult substances, were measured by HPLC (Carvalho *et al.*, 1985; Guilhermino *et al.*, 1996). For all the other chemicals, nominal concentrations were used.

Chemicals. Metallic compounds, SDS, methanol, and ethanol were purchased from Merck (Germany), DBS was a gift of Unilever UK Central Resources Ltd., parathion was from BDH (England), paraoxon was from Sigma (St. Louis, MO), chlorpyrifos was from Riedel-de Haën (Germany), and DCA from Aldrich (England). All chemicals were at least 95% pure.

Data analysis. LC_{50} values to *D. magna* and respective 95% confidence limits were determined by probit analysis (Finney, 1971). The Pearson product–moment correlation coefficient was used to test the association between the oral LD_{50} to the rat and both 24-h LC_{50} and 48-h LC_{50} to *D. magna* using 15 compounds as test substances. Since there was evidence of a strong relationship, a revision of the literature values for rat oral LD_{50} values and *D. magna* 24-h LC_{50} values was performed, and 54 data points were further used to ascertain the relationship between *D. magna* LC_{50} values and the acute toxicity to the rat based on the EU classification of chemicals. Two groups were formed: a group of both very toxic and toxic chemicals (oral $LD_{50} < 200$ mg/kg) and a group of chemicals considered harmful or unclassified (oral $LD_{50} \geq 200$ mg/kg). A logistic regression model was then used to determine the "best" cutpoint in the 24-h LC_{50} values that could predict the toxicity to the rat (Hosmer and Lemeshow, 1989). This cutpoint can then be considered as a confirmation test for rat toxicity, and its criterion validity (Abramson, 1984) is also discussed, using as criteria the sensitivity, specificity, and relative risk.

RESULTS

LC_{50} values of 15 compounds for *D. magna* determined in this study and the corresponding oral LD_{50} values for the rat found in the literature are listed in Table 1. For these chemicals, the correlation coefficients between LD_{50} values for the rat and both 24- and 48-h LC_{50} values for *D. magna* (in logarithm scale to improve the assumption of normality) were highly significant. The value of r was 0.93 using *D. magna* 24-h LC_{50} values and 0.91 using *D. magna* 48-h LC_{50} values. With the data in Table 2, a linear regression was used to model the probability of toxicity to the rat using as predictor the 24-h LC_{50} values for *D. magna* for the 54 chemicals in Table 2. The value of r was low (0.478), and for this reason, a logistic regression model was then used. As judged by the goodness-of-fit χ^2 P value (0.19), the model fit the data. Figure 1 is the logistic curve, the value of $\ln(LD_{50})$ that serves as a "test" to predict the probability of toxicity to the rat (-1.50) and the chemicals the toxicity of which is wrongly predicted by the model. Two of them are false positives (predicted as toxic and actually classified as harmful) and 11 are false negatives (predicted as not toxic and actually classified as toxic).

Using 0.22 mg/L as text value, the criterion validity of *D. magna* 24-h LC_{50} to predict toxicity to the rat is presented in Table 3. The *D. magna* test has a high specificity, correctly identifying 93.9% of the chemicals that are nontoxic to the rat, and consequently a low false-positive rate (2/12). Nevertheless, its sensitivity is rather low, indicating toxicity in only 47.6% of the chemicals that are in fact toxic to the rat. An overall measure of validity, which takes account of both toxic and nontoxic substances to the rat, is

TABLE 1

LC_{50} Values at 24 and 48 h (mg/L) and Respective 95% Confidence Limits of 15 Chemical Compounds Determined in This Study for *D. magna* and the Corresponding Oral LD_{50} (mg/kg) Values for the Rat

Chemical	*D. magna* LC_{50} (mg/L) 24 h	*D. magna* LC_{50} (mg/L) 48 h	Rat oral LD_{50} (mg/kg)
Paraoxon	0.00055 (0.00052–0.00057)	0.00019 (0.00018–0.00020)	1.8[a]
Parathion	0.00219 (0.00217–0.00221)	0.00216 (0.00215–0.00217)	13[b]
DCA	0.271 (0.269–0.274)	0.100 (0.099–0.101)	648[c]
Chlorpyrifos	*	0.344 (0.343–0.345)	145[c]
Mercurous chloride	0.0027 (0.0026–0.0028)	0.002 (0.0019–0.0021)	37[a]
Cadmium chloride	0.071 (0.032–0.159)	0.017 (0.0166–0.0174)	88[a]
Copper sulfate	0.399 (0.391–0.406)	0.0826 (0.0823–0.0829)	960[a]
Zinc sulfate	35.403 (28.360–44.192)	4.029 (3.999–4.056)	2,150[a]
Sodium dichromate	1.854 (1.813–1.895)	0.778 (0.777–0.779)	160[d]
Chromous chloride	40.507 (33.980–48.287)	21.531 (18.957–24.456)	1,870[a]
Sodium bromide	15,322 (14,613–16,065)	7451 (7394–7508)	3,500[a]
Ethanol	9,788 (7,387–12,970)	5,680 (4364–7392)	13,700[e]
Methanol	4,816 (3,616–6,414)	3,289 (2461–4395)	13,000[e]
SDS	45.898 (45.697–46.101)	19.129 (19.023–19.235)	1,288[a]
DBS	38.514 (37.452–39.605)	9.546 (9.491–9.600)	2,000[a]

[a]Merck (1989).
[b]Gaines (1969).
[c]Merck, personal communication.
[d]Anger *et al.* (1986).
[e]NIOSH (1977).
*It was not possible to calculate the LC_{50} at 24 h.

the relative risk of toxicity to the rat, which is 3.2 with a 95% confidence interval from 1.7 to 6.3. This means that the risk of toxicity to the rat is 3.2 higher for chemicals with a *D. magna* $LC_{50} < 0.22$ mg/L than for chemicals with a *D. magna* $LC_{50} \geq 0.22$ mg/L.

DISCUSSION

This study was assigned to ascertain the relationship between the LC_{50} values of 54 chemicals for *D. magna* and the corresponding LD_{50} values for the rat to contribute to the investigation of the possibility of using invertebrate tests as prescreening methods for assessment of the toxicity of new chemicals for classification and labeling purposes.

TABLE 2

Acute Toxicity of 54 Chemical Compounds to *D. magna* (24-h LC_{50} Values) and to the Rat (Oral LD_{50} Values)

Chemical	*D. magna* LC_{50} (mg/L)	Rat LD_{50} (mg/kg)
Acetic acid	10.79[a]	375[m]
Amitriptyline	1.151[g]	380[i]
Amphetamine sulfate	60.434[g]	55[i]
Aniline	0.9[b]	440[i]
Arsenic trioxide	7.5[g]	15.1[i]
Aspirin	1,468.2[g]	1,500[i]
Cadmium chloride	0.071*	88[i]
Caffeine	683.7[g]	355[i]
Carbon tetrachloride	69.37[a]	2,800[m]
Chloroform	64.23[a]	800[m]
Chlorpyrifos	0.0037[s]	145[i]
Chromous chloride	40.501*	1,870[i]
Copper chloride	0.172[a]	140[m]
Copper sulfate	0.399*	960[i]
DBS	38.514*	2,000[i]
DCA	0.271*	648[k]
Diazepan	4.271[g]	710[i]
Diazinon	0.0009[d]	235[q]
Dichlorvos	0.00006[c]	80[o]
Digitoxin	24.21[g]	33.35[r]
Disulfoton	0.055[c]	6.8[i]
Endosulfan	0.620[f]	43[o]
Ethanol	9,788*	13,700[m]
Ethylene glycol	4,8582[g]	4,698.7[r]
Fenitrothion	0.0002[c]	250[i]
Ferrous chloride	74.41[a]	29.74[m]
Ferrous sulfate	14.28[g]	319[r]
Formaldehyde	57[h]	800[i]
Hexachlorophene	0.1982[g]	66[i]
Isopropanol	6,850.26[g]	5,041.6[r]
Lindan	14.5[g]	91[o]
Malathion	0.354[g]	1375[j]
Mercurous chloride	0.0027*	37[m]
Methanol	4,816*	13,000[m]
Methyl parathion	0.00000031[e]	14[j]
Paraoxon	0.00055*	1.8[j]
Parathion	0.002189*	13[j]
p-chloroaniline	13[b]	340[n]
p-cresol	14[b]	1,800[i]
Pentachlorophenol	0.44[a]	50[m]
Phenobarbital	1,400.3[g]	162[i]
Phenol	9.129[g]	530[i]
p-Nitrophenol	11[b]	616[i]
Quinine sulfate	44.8[g]	455.8[i]
SDS	45.898*	1,288[i]
Sodium bromide	15,322*	3,500[i]
Sodium chloride	1,022.6[a]	3,000[m]
Sodium dichromate	1.854*	160[l]
Sodium fluoride	307.7[g]	180[i]
Stannous chloride	60.8[a]	700[m]
Thallium sulfate	8.1[g]	25[i]
Thiometon	5.49[c]	70[p]
Toluene	8.0[h]	7,530[i]
Zinc sulfate	35.403*	2,150[i]

[a]Khangarot and Ray (1988).
[b]Kühn *et al.* (1989).
[c]Gälli *et al.* (1994).
[d]Fernández-Casalderrey *et al.* (1994).
[e]Fernández-Casalderrey *et al.* (1995).
[f]Fernandez *et al.* (1993).
[g]Lilius *et al.* (1994).
[h]Janssen and Persoone (1993).
[i]Merck (1989).
[j]Gaines (1960).
[k]Merck, pers. communication.
[l]Anger *et al.* (1986).
[m]NIOSH (1977).
[n]Marty and Nepierre (1979).
[o]Gaines (1969).
[p]Eto (1974).
[q]Gasser (1953).
[r]Ekwal *et al.* (1989).
[s]Kersting and Van Wijngaarden (1992).
*This study.

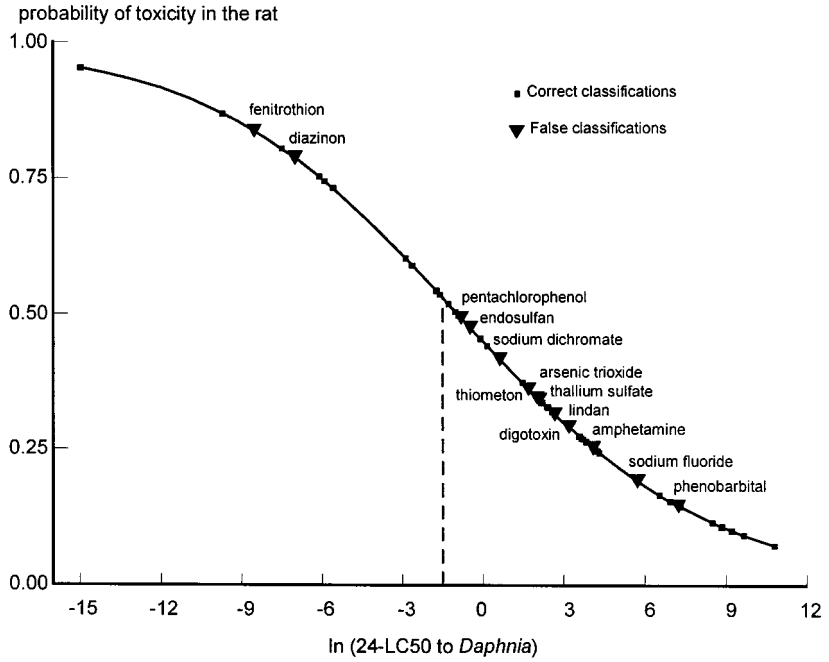

FIG. 1. Logistic curve for predicting toxicity to the rat based on *D. magna* 24-h LC_{50}, indicating the cutpoint, false positives, and false negatives.

To summarize its validity, the *D. magna* test is more specific than sensitive for an indication of toxicity to the rat. When it is used for a chemical that has a high probability of being very toxic to *D. magna* ($LC_{50} < 0.22$ mg/L), the test provides considerable information if it is positive, virtually giving evidence of toxicity to the rat (with a probability of 0.83). On the other hand, a negative test (*D. magna* $LC_{50} > 0.22$ mg/L) would not be so reliable in ruling out toxicity to the rat; the probability of correctly assigning nontoxicity to the rat is 0.74. Therefore, the test with *D. magna* gives more accurate results for chemicals with a *D. magna* LC_{50} lower than 0.22.

TABLE 3
Criterion Validity of *D. magna* 24-h LC_{50} for Prediction of Chemical toxicity to the Rat

	Rat		
Daphnia (test)	Toxic	Nontoxic	Total
$LC_{50} < 0.22$ mg/L	10	2	12
$LC_{50} \geq 0.22$ mg/L	11	31	42
Total	21	33	54
Sensitivity = 10/21 = 47.6%			
Specificity = 31/33 = 93.9%			
Predictive value of a toxic *Daphnia* value ($LC_{50} < 0.22$ mg/L) = 10/12 = 83.3%			
Predictive value of a nontoxic *Daphnia* value ($LC_{50} \geq 0.22$ mg/L) = 31/42 = 73.8%			

The false-positive results were obtained for two organophosphate insecticides, fenitrothion and diazinon. The failure of this test is not surprising, as these compounds have high selectivity against insects and are known to have relatively low toxicity to mammals. Selective pesticides are examples of substances that may cause problems in the extrapolation of mammal toxicity based on invertebrate tests. However, these chemicals are derived from molecules with known active mechanisms. Thus, their toxicity could be predicted from chemical structure. False-negative classifications were obtained for 11 chemicals, including metallic compounds, organochlorine insecticides, and other organic compounds. The toxicity of these compounds to the rat, wrongly predicted by the test, demonstrates that for LD_{50} values greater than 0.22 the test does not perform so well, but still has a high predictive value (73.8%). These results are in good agreement with those reported in the literature (Khangarot and Ray, 1988; Khangarot *et al.*, 1987).

As far as validity is concerned the test performs quite well. But validity is not the only issue in question. There are ethical issues to be taken into consideration and there is no doubt that rats (and other mammals commonly used in toxicity testing) are very sensitive to pain and distress. Routine use of the *D. magna* test as a prescreening method will greatly reduce the number of mammals required for regulatory acute oral toxicity testing. In addition to ethical considerations, the cost effectiveness of this procedure is evident since it has a high predictive value to rat. LD_{50} values and daphnids are less expensive and require less

space and human care than rats. Furthermore, *D. magna* is a standard organism in ecotoxicology and the acute toxicity test with this species is standardized by international organizations such as EU (1992) and EPA (1991). Using the logistic regression model, the *D. magna* test seems to have a predictive capacity comparable to that of mammalian cytotoxicity tests. In addition, it is an *in vivo* test taking into account the biotransformation of toxicants and potential integrated effects that occur in the organism as a whole, and a considerable number of LC_{50} values for a great variety of chemical agents already exist. Thus, use of *D. magna* results could be advantageous at least in some situations.

CONCLUSION

The results of this study and data published in the literature provide good evidence of the applicability of the *Daphnia magna* acute test as a prescreening method in toxicity testing. Exploration of the toxicity data available in the literature for this invertebrate and mammals is necessary to overcome the barriers regarding the acceptance of this alternative.

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