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Short Communication

ACUTE AND CHRONIC EFFECTS OF DIPHENHYDRAMINE AND SERTRALINE MIXTURES IN *CERIODAPHNIA DUBIA*

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Abstract: *Ceriodaphnia dubia* were tested to evaluate the acute and chronic interactive effects of diphenhydramine and sertraline. Observed effects were compared with 2 reference toxicity models, the concentration addition model and the independent action model. Results indicate that the 2 drugs exhibit additive toxicity in *C. dubia*. In some cases, individually sublethal concentrations of the chemicals resulted in 100% mortality when combined, demonstrating the potentially severe impact of trace environmental contaminants. *Environ Toxicol Chem* 2013;32:xx–xx. © 2013 SETAC

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INTRODUCTION

In recent years, trace amounts of pharmaceuticals have been detected in surface waters throughout the United States [1]. However, little information exists regarding the effects these chemicals may have on the environment. It has been widely assumed that low concentrations (<1 μg/L) pose little or no threat to aquatic organisms, but recent studies indicate the potential for certain chemicals to adversely affect the health of ecosystems [2]. Particularly, drugs that are active on the central nervous and endocrine systems may cause significant physiological changes in aquatic organisms [3]. Additionally, the effects of multiple drugs with similar mechanisms of action may result in additive or synergistic toxicity [4]. The complexity of drug interactions and the lack of data regarding the effects of pharmaceuticals on aquatic organisms make it difficult to anticipate how aquatic environments may be affected by the presence of these contaminants [2].

Selective serotonin reuptake inhibitors are a class of prescription drugs prescribed to manage conditions such as depression, anxiety, obsessive–compulsive disorder, and other psychiatric pathologies. Sertraline (Zoloft; Pfizer) is one of the most frequently prescribed selective serotonin reuptake inhibitors in the United States. In humans, selective serotonin reuptake inhibitors cause an increase in extracellular serotonin concentrations by blocking reuptake into neurons at the synaptic cleft. Diphenhydramine (Benadryl; McNeil-PPC) is a widely used over-the-counter antihistamine. The primary mechanism of action for diphenhydramine is antagonism of the histamine H_1 receptor, which blocks histamine from binding to receptors and reduces histamine-mediated allergic responses. Diphenhydramine is also an inhibitor of serotonin reuptake at the synaptic cleft [5], although it is not prescribed as an antidepressant.

Research in the area of combined chemical toxicity is necessary to anticipate how multiple pharmaceuticals may affect aquatic environments. Diphenhydramine and sertraline, both of which have been documented to be present in surface waters at concentrations up to 0.1 μg/L [6–10], were selected for the present study due to their pharmacologically distinct properties yet similar mechanisms of action through inhibition of serotonin reuptake. The objective of the present study was to assess the acute and chronic effects of combined diphenhydramine and sertraline exposure to *Ceriodaphnia dubia*. Acute mortality and reproductive effects of sertraline and diphenhydramine were assessed and compared to predicted effects by 2 joint action reference models, the concentration addition model and the independent action model. The concentration addition model assumes a similar mechanism of action for toxicants, so individual effects from chemicals are assumed to contribute to toxic effects additively when combined [4,11,12]. The independent action model assumes dissimilar mechanisms of action in which toxic effects from chemicals act independently, hypothetically resulting in a subadditive response [4,13–15].

MATERIALS AND METHODS

Toxicity tests

Ceriodaphnia dubia neonates were obtained from Aquatic Biosystems and were acclimated for approximately 1 mo prior to testing. Organisms were maintained in a stock culture consisting of 60 organisms cultured individually in 30-mL plastic cups filled with 15 mL moderately hard water, changed daily. In accordance with US Environmental Protection Agency guidelines for *C. dubia* culture maintenance and toxicity experiments [16,17], the culture was maintained in an incubator at 25 °C with a 16:8-h light:dark photoperiod, and each individual was fed daily with 100 μL of a mixture of yeast, cerophyl, and trout chow (Aquatic Biosystems) and 100 μL algae (*Selenastrum* spp.; Aquatic Biosystems). Moderately hard water was prepared with 1.20 g $MgSO_4$, 1.92 g $NaHCO_3$, 0.080 g KCl, and 1.20 g $CaSO4 \cdot 2H_2O$ added to 20 L Milli-Q water [16,17], which was aerated and conditioned for 48 h prior to use. Water-quality parameters were assessed prior to use of moderately hard water to ensure that the following ranges were met: pH of 7.9 to 8.4, hardness of 80 mg/L to 90 mg/L (as $CaCO_3$), and alkalinity of 60 mg/L to 80 mg/L (as $CaCO_3$). The fecundity of each

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individual was recorded daily, and a new stock culture was initiated from the neonates of every fourth brood. The culture was allowed to acclimate to laboratory conditions for 5 generations prior to initiation of experiments. Culture quality and sensitivity were verified with acute 48-h reference toxicity tests using $CuSO_4$, and acute median lethal mortality for $CuSO_4$ was within acceptable laboratory ranges (34.8 μg Cu^{2+}/L).

Preliminary 48-h acute and 7-d chronic tests were performed to determine appropriate concentration ranges for experiments. For acute experiments, 117 combinations of concentrations of mixtures of diphenhydramine (0.0–4.5 mg/L) and sertraline (0.0–0.6 mg/L) were prepared with 4 replicates per treatment. For chronic tests, 24 combinations with varying diphenhydramine (0.0–3.0 mg/L) and sertraline (0.0–0.5 mg/L) concentrations were prepared with 10 replicates per treatment. Neonates younger than 8 h were collected from third or fourth broods and pipetted into 30-mL plastic cups, each containing 15 mL test solution (moderately hard water with concentrations of diphenhydramine and sertraline based on the treatment group); 100 μL yeast, cerophyl, and trout chow; and 100 μL algae (*Selenastrum* spp.). For acute tests, each cup received 5 to 8 neonates (total number of *C. dubia* individuals = 2466), and mortality (indicated by lack of movement within 30 s of observation) was recorded after 48 h. For chronic tests, each cup received a single neonate (total number of *C. dubia* individuals = 240), and the number of living offspring produced by each individual was recorded daily until more than 60% of the controls had a third brood [17].

Statistical analyses

Observed and predicted concentration–response relationships were modeled for acute mortality and chronic reproductive effects using logistic regressions in SAS (PROC LOGISTIC, Ver 9.2; SAS Institute) according to the following formula

$$\text{logit}(y) = mx + b \tag{1}$$

Logit(y) is the log of the probability of the expected effect (mortality for acute tests, total offspring number for chronic tests), and m, x, and b represent the slope of the regression, the concentration of the toxicant, and the regression intercept value, respectively. The probability of the expected effect, y, for any concentration, x, was calculated using the following formula:

$$y = \frac{e^{mx+b}}{(1 + e^{mx+b})} \tag{2}$$

Acute and chronic median lethal and effective concentrations (LC50 and EC50, respectively) for diphenhydramine and sertraline were estimated using logistic regressions generated from each chemical individually. Fieller's procedure was used to calculate 95% confidence intervals for each LC50 and EC50 value [18]. Concentrations for each chemical were converted into toxic units based on LC50 or EC50 values for individual toxicants [15]. Toxic units are calculated by dividing the concentration for a specific toxicant (c_i) by the EC50 (or LC50)

$$\text{toxic units} = \frac{c_i}{EC50_i} \tag{3}$$

Using toxic units, logistic regressions were generated to model observed mortality and reproduction. The observed regressions were then compared using a likelihood ratio test to compare observed effects to predicted effects by the concentration addition model and the independent action model [19]. The concentration addition model [4,11,12], defined below, assumes *similar* mechanisms of toxicity between mixtures and states that for n chemicals exhibiting additive toxicity, the quotients of concentration c for chemical i and the concentration of chemical i that produces an x% response when applied individually sum to 1

$$\sum_{i=1}^{n} \frac{c_i}{\text{EC}x_i} = 1 \tag{4}$$

The independent action model [4,13–15] assumes *dissimilar* mechanisms of toxicity and states that for n chemicals, the product of each chemical's individual probability of nonresponse (1–$E[x_i]$) subtracted from 1 equals the expected response to a mixture of the chemicals $E(x_{1,2\ldots n})$

$$E\,(x_{1,\,2,\,\ldots n}) = 1 - \prod_{i=1}^{n} (1 - E(x_i)) \tag{5}$$

All models were compared using Wald 95% confidence intervals, which were calculated by multiplying the standard errors of regression coefficients by $z_{0.025}$, the 97.5th percentile point of the normal distribution (~1.96).

RESULTS AND DISCUSSION

Logistic regressions of observed data significantly explained *C. dubia* acute and chronic effects in response to diphenhydramine and sertraline exposures (Wald χ^2, $p < 0.0001$). Acute 48-h LC50 values (95% confidence interval) for diphenhydramine and sertraline were determined to be 3.94 mg/L (3.77–4.15 mg/L) and 0.433 mg/L (0.417–0.449 mg/L), respectively. Chronic EC50 values (95% confidence interval) for reproduction were determined to be 0.991 mg/L (0.525–1.52 mg/L) for diphenhydramine and 0.184 mg/L (0.101–0.274 mg/L) for sertraline. These results are comparable to results from similar studies involving *C. dubia* and *Daphnia magna* [3,20,21], with the exception of the 48-h acute LC50 for diphenhydramine in *D. magna*, which Berninger et al. [8] determined to be 0.374 mg/L, approximately 1 order of magnitude lower than diphenhydramine acute LC50 values for *C. dubia* (3.94 mg/L). This discrepancy in sensitivities may be indicative of differences in sensitivities or distinct mechanisms of toxicity between the 2 species.

Observed and predicted (both concentration addition and independent action) concentration–response relationships were modeled with logistic regressions (Figure 1). Quantitative comparisons of regressions using likelihood ratio tests for acute mortality showed that all 3 regressions differed significantly from one another (Table 1). While neither the concentration addition nor the independent action model significantly predicted acute mortality, the observed regression fell between the regressions for the 2 predictive models, possibly reflecting the drugs' overlapping pharmacological properties (inhibition of serotonin reuptake) as well as each chemical's distinct mechanism of action. However, the ability of either model to predict toxic effects resulting from mixtures of different substances does not necessarily support the hypotheses of similar or dissimilar mechanisms of toxicity respectively assumed by the models [14]. Furthermore, differences detected by statistical tests should be interpreted with consideration to biological relevance. For chronic tests, the observed regression was not significantly different from either the concentration addition or the independent action model (Table 1).

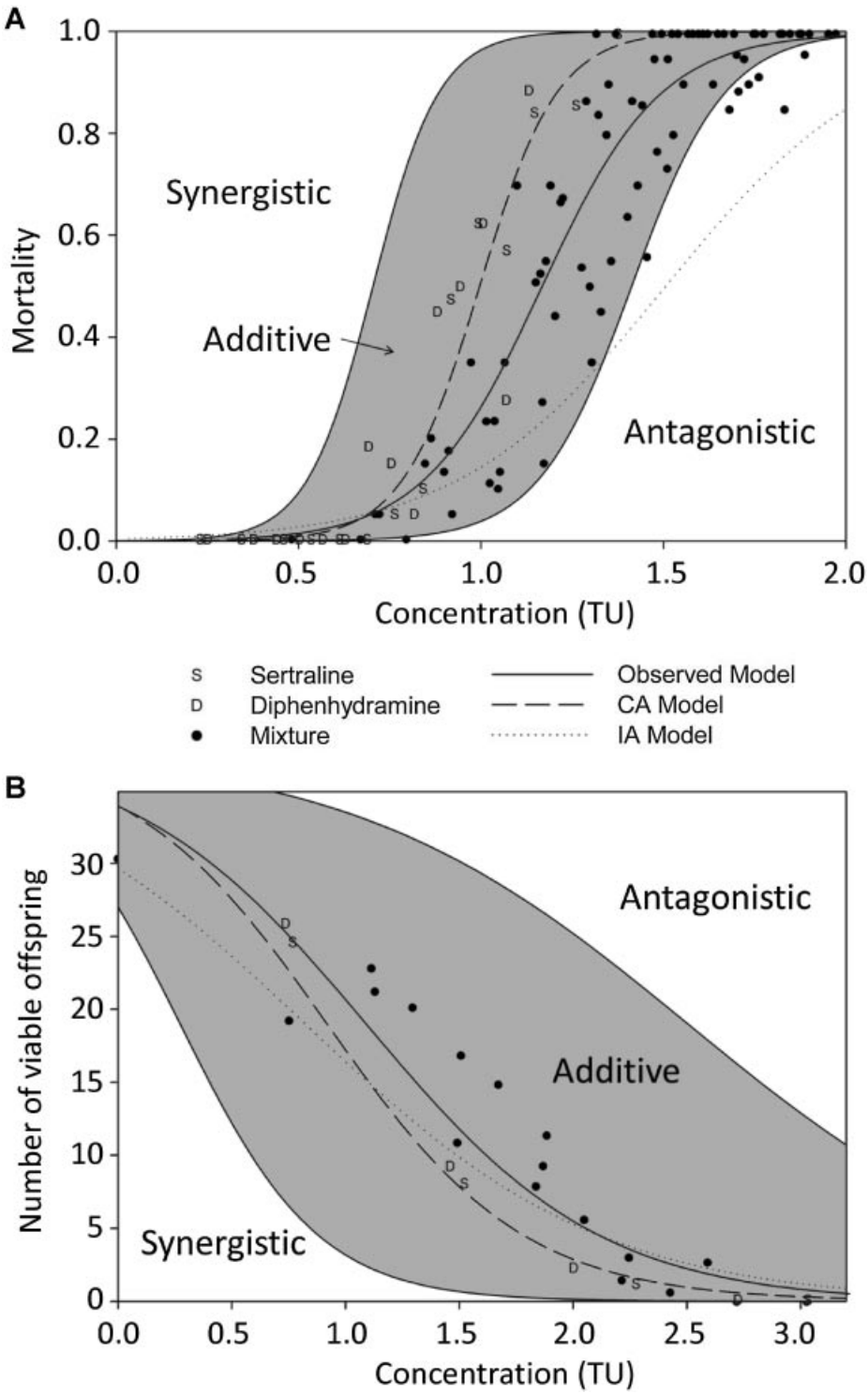

Figure 1. Logistic regressions of observed and predicated (concentration addition [CA] and independent action [IA]) concentration–response relationships for (**A**) 48-h acute *Ceriodaphnia dubia* mortality and (**B**) 7-d reproduction in response to individual and mixed concentrations of diphenhydramine and sertraline. Shaded regions indicate 95% confidence interval for additive model (CA). Regions outside of the concentration addition confidence interval are designated as synergistic or antagonistic; regions within the concentration addition confidence interval are additive. Concentrations are given in toxic units (TU). For acute mortality, 1 toxic unit = 3.94 mg/L diphenhydramine = 0.433 mg/L sertraline. For chronic reproduction, 1 toxic unit = 0.991 mg/L diphenhydramine = 0.184 mg/L sertraline. Observed data points represent the mean effect for each treatment (acute $n = 4$, chronic $n = 10$).

To qualitatively distinguish deviations from additive toxic interactions (synergism or antagonism), 95% confidence intervals for the concentration addition model were used as reference boundaries for interactive effects. Observed regressions for both acute and chronic experiments fell completely within the additive region (Figure 1). Although no synergistic toxicity was observed, the highest concentrations that produced no acute mortality for single-compound exposures of diphenhydramine (2.5 mg/L) and sertraline (0.3 mg/L) resulted in 100% mortality when combined. These results indicate that apparently nontoxic concentrations of nonsynergistic chemicals can drastically affect the health of exposed organisms when exposed

Table 1. Likelihood ratio contrasts of regression models for observed *Ceriodaphnia dubia* and concentration addition (CA) and independent action (IA) models[a]

	Observed vs CA		Observed vs IA	
	χ^2	p	χ^2	p
Acute	14.35	0.0002	87.81	<0.0001
Chronic	0.44	0.5095	0.19	0.6619

[a] Regression contrasts with $p \leq 0.05$ are considered significantly different ($df = 1$).

in combination. Furthermore, the interactive effects of the hundreds of complex combinations of trace chemicals that are present in surface waters in the United States remain untested, although many of these mixtures may result in additive toxicity and some have been found to act synergistically in aquatic organisms [1,2,4,22–24]. More research is needed to identify the overall ecological impact of multiple trace contaminants.

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