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Chronic Toxicity of Diphenhydramine Hydrochloride and Erythromycin Thiocyanate to Daphnia, *Daphnia magna*, in a Continuous Exposure Test System

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Abstract Diphenhydramine hydrochloride (DH; Benadryl™, an over-the-counter antihistamine) and erythromycin thiocyanate (ET; a commonly used macrolide antibiotic) are pharmaceutical compounds whose chronic toxicity to *Daphnia magna* had not been characterized. Continuous exposure to DH concentrations about 5 times greater than the maximum reported environmental concentration of 0.023 μg/L for 21 days or to ET concentrations about 40 times the maximum reported environmental concentration of 6 μg/L for 21 days did not significantly impact *D. magna* survival and production. In this study the no observable effect concentration for DH was 0.12 μg/L and for ET was 248 μg/L.

Keywords Toxicity of diphenhydramine hydrochloride and erythromycin thiocyanate to *Daphnia* · Toxicity of pharmaceutical compounds to an aquatic invertebrate

Pharmaceuticals and personal care products (PPCP) are a group of compounds that include prescription and over-the-counter pharmaceutical compounds, detergent by-products, fragrances, cosmetics, sunscreen agents, and diagnostic agents. These compounds are continually introduced into the aquatic environment from a number of sources including wastewater treatment plants (WWTP). Loss of PPCP from the environment can occur by biodegradation, hydrolysis, photolysis, and other processes. The loss of some of these compounds is countered by continual replenishment from WWTP and the other sources, effectively sustaining multi-generational life-long exposures for aquatic organisms. The ramifications of the long-term persistence of PPCP, especially pharmaceutical compounds, in aquatic environments, and the effects that those compounds have on aquatic organisms are largely unknown.

Kolpin et al. (2002) documented the presence of many PPCP compounds or their degradation products in surface waters across the United States. Pharmaceutical compounds designed to specifically modify physiological processes in humans and cultured animals may also affect non-target aquatic invertebrate populations. Two dissimilar, yet relatively common pharmaceutical compounds (an antihistamine and a macrolide antibiotic) were the focus of this chronic toxicity study. The study objective was to determine if chronic exposure to diphenhydramine hydrochloride (DH; Benadryl™, one of several trade names; mention of trade or manufacturer name is solely for providing specific information and does not imply endorsement by the US Geological Survey) and erythromycin thiocyanate (ET) concentrations greater than their environmental concentrations would have an effect on *Daphnia magna* survival, reproduction, and growth during chronic exposure.

Materials and Methods

Diphenhydramine hydrochloride [Chemical Abstract Service (CAS) number, 147-24-0; molecular weight, 291.82] was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). The chemical purity determined by thin layer chromatography was

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≥98%. Erythromycin thiocyanate (CAS number, 7704-67-8; molecular weight, 793.02; potency = 791 μg/mg as is, 833 μg/mg anhydrous) was manufactured by Abbott Laboratories (Abbott Park, IL) and acquired from Bimeda, Inc. (La Seur, MN).

A test chemical stock solution was prepared at intervals of 4 days or less by quantitatively transferring DH to a 500 mL volumetric flask with 100 mL of deionized water (water deionized to a specific resistance of >17.8 mΩ/cm with a Barnstead E-pure water purification system, Dubuque, IA). Erythromycin thiocyanate was transferred to the same flask with 100 mL of acetonitrile [high pressure liquid chromatography (LC) grade, Fisher Scientific, Pittsburg, PA]. The flask volume was adjusted to 500 mL with deionized water. Analytical standards used to generate LC-mass spectrometer (MS) calibration curves were prepared by diluting the stock solution with deionized water.

Water samples for determining test chemical concentrations were prepared for analysis by filtration through a conditioned polypropylene syringe filter (Acrodisc® GHP, 13 mm, 0.2 μm; Pall Life Sciences, East Hills, NY). Conditioning was required because of an interfering compound residing on the filters. Conditioning involved forcing about 3 mL of methanol (LC grade, Fisher Scientific) through each filter followed by about 3 mL of acetone (LC grade, Fisher Scientific), about 3 mL of a 10% glacial acetic acid (LC grade, Fisher Scientific): 20% acetone solution prepared in deionized water, and finally about 6 mL of deionized water.

Identification and quantification of the test chemicals were performed using an Agilent 1100 LC system (Agilent Technologies, Inc., Santa Clara, CA) with a Waters XBridge™ C18, 3.5 μm, 2.1 × 150 mm analytical column (Waters Corporation, Milford, MA) and an Agilent model G1946D mass selective detector. The LC parameters included an isocratic mobile phase of 80% solvent A (74.9% water, 25% acetonitrile, and 0.1% acetic acid) and 20% solvent B (99.9% acetonitrile and 0.1% acetic acid), a flow rate of 0.3 mL/min, an injection volume of 100 μL, a column temperature of 30°C, and a run time of 8 min. Mass spectral detection was achieved using atmospheric pressure electrospray ionization in the positive mode. Mass spectrometer operating parameters included a capillary voltage of 3,250 V, a nebulizer pressure of 25 psig, a drying gas (nitrogen) flow rate of 10 mL/min, and a gas temperature of 350°C. The select ion monitoring parameters are presented in Table 1. Programmed capillary exit voltage (fragmentor voltage) changes were used to produce sufficient fragmentation and characteristic fragments for each compound. The acceptable difference between the quantitation and confirmation ions retention times was ≤0.03 min.

Table 1 Instrumental parameters for determining diphenhydramine hydrochloride (DH) and erythromycin thiocyanate (ET) concentrations in water using a high pressure liquid chromatography-mass spectrometry system under select ion monitoring conditions (electrometer gain, 2 V; tolerance, 20%)

Production	Fragmentor voltage	Ion assignments (*m/z*)	Confirmation/ quantitation ions ratio
DH quantitation	140	256.1[a]	89
DH confirmation	140	167.1	
ET quantitation	140	734.4[a]	8.9
ET confirmation	200	576.3	

[a] mass + hydrogen

The concentrations of the test chemicals in each sample were determined from the chemical's peak area and a quadratic regression equation developed from 6 analytical standard solutions with DH concentrations ranging from 0.04 to 1.4 μg/L and with ET concentrations ranging from 0.1 to 4 μg/L. The method quantitation limits for DH and ET were 0.047 and 0.093 μg/L, respectively.

The *D. magna* culture was maintained in the following conditions: water temperature, 20°C; lighting, 16 h of incandescent light and 8 h of darkness with 20 min transition periods for lights to fade on and off. The exposure system was described in Meinertz et al. (2008). Test chemical working solutions were delivered to the stream of well water using MasterFlex® Digi-Staltic® model 77310-01 pump drives, MasterFlex® Easy-Load® II model 77202-60 heads, and a model 77310-02 controller (Cole-Parmer Instrument Co., Vernon Hills, IL). Working solutions were prepared every 4 days.

Eight treatment groups were assigned one of the following exposure designations: control (no chemical exposure), 0.12 μg/L DH, 62 μg/L DH, 620 μg/L DH, 0.60 μg/L ET, 300 μg/L ET, 3,000 μg/L ET, and 62 μg/L DH: 300 μg/L ET. Each of the 8 treatment groups consisted of seven test chambers with one *D. magna* per chamber. Each chamber within the exposure system (56 chambers total) was randomly assigned to a treatment group.

Water and test chemical flow through the exposure system were initiated 2 days before stocking the test system with *D. magna*. On Day 0 (first day of exposure), 1 < 24-h old *D. magna* was randomly assigned to 1 test chamber. Chambers were inspected each day for live first generation *D. magna* and offspring production. Young were enumerated if present. A daily water sample (1 mL) was collected from each chamber before feeding and water from common treatment groups pooled in 20-mL glass vials. Pooled water from the control and low concentration treatment groups were not diluted. Pooled water from the mid range concentration treatment groups were diluted 1:100 with deionized water. Pooled water from the high

concentration treatment groups were diluted 1:1,000 with deionized water. All samples were forced through conditioned Acrodisc® GHP syringe filters into LC vials.

D. magna food (100 µL) was dispensed into each test chamber, twice daily during weekdays, at an interval of more than 5 h, and once each weekend day. Daily dissolved oxygen concentrations ranged from 6.3 to 8.2 mg/L. Daily ph ranged from 7.2 to 7.6. Daily temperature ranged from 19.7 to 20.9°C. Flow rates through test chambers were about 2.6 mL/min producing about 19 volume exchanges per 24 h. Weekly measurements of alkalinity and water hardness ranged from 100 to 125 mg/L as $CaCO_3$ and 154 to 174 mg/L as $CaCO_3$, respectively. Weekly light intensity measurements directly over the chambers ranged from 85 to 177 lux during the 16 h periods of light.

First generation *D. magna* that survived through the 21-day trial were transferred to a 4% formalin-sucrose solution (Haney and Hall 1973). A wet mount of each preserved *D. magna* was used to determine length (mm) with a digital camera (model Coolpix 5000; Nikon Corporation, Japan) mounted on a microscope (model E600; Nikon Corporation, Japan) and imaging software (Image Pro Express, version 4.5, Media Cybernetics, Silver Spring, MD). Length was measured from the top of the head to the base of the spine.

Concentration was considered to be a categorical versus continuous variable in data analyses because a limited number of concentrations were used. A Kaplan-Meier test of homogeneity of survival time across concentration (Kaplan and Meier 1958) was used to assess differences in times to death.

Body length data as a function of concentration were fit to a generalized linear model (McCullagh and Nelder 1989); likelihood-ratio F-tests were used to test hypotheses about the relation between body length and concentration.

The times to first brood could only be observed in *D. magna* that survived long enough to reproduce, therefore adult *D. magna* that died before having a brood were excluded from the analysis. A Kaplan-Meier test of homogeneity was used to analyze the effect of concentration on time to first brood.

The number of broods and total number of young are non-negative integer-valued counts where a Poisson distribution is assumed. A generalized linear model was developed to assess the effect of concentration on the number of broods and total number of young produced.

The model parameters were estimated using maximum-likelihood methods and likelihood-ratio Chi-square tests were used to test hypotheses about the relation between the counts and *C*. Statistical analyses were performed with SAS software (SAS 2004) and analyses considered to be significant if $p \leq 0.05$.

Results and Discussion

Diphenhydramine hydrochloride is an over-the-counter antihistamine, sedative, and hypnotic that has been reported to be present in raw, untreated drinking water samples taken throughout the United States (Focazio et al. 2008). Focazio et al. (2008) reported a maximum concentration of 0.023 µg/L. To our knowledge, there were no data describing the toxicity of DH to aquatic invertebrate organisms available in the scientific literature.

Erythromycin thiocyanate, an antibiotic macrolide, is one of several erythromycin salts that have been used for the treatment and prevention of human and animal diseases for several decades. Erythromycin has been reported to be in a variety of environmental water samples in a number of different countries. In a survey of 139 streams spread across the US, Koplin et al. (2002) reported finding erythromycin in 30 samples with a maximum concentration of 1.7 µg/L. Also in the United States, Focazio et al. (2008) reported erythromycin in untreated drinking water with a maximum concentration of 0.3 µg/L and Karthikeyan and Meyer (2006) reported maximum concentrations in wastewater treatment plant influents and effluents as 1.2 and 0.3 µg/L, respectively. Other reports of erythromycin in aquatic environments included a survey of sewage treatment plant effluents and surface waters in Germany (Hirsch et al. 1999). Maximum erythromycin concentrations in sewage treatment plant effluents were 6 µg/L and in surface waters were 1.7 µg/L. Ashton et al. (2004), investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom, reported a maximum erythromycin concentration upstream from a sewage treatment plant as 0.057 µg/L, in the sewage treatment plant effluent as 1.8 µg/L, and downstream from the plant as 1.0 µg/L.

Data describing the toxicity of ET to aquatic invertebrate organisms were primarily limited to acute studies (Dojmi di Delupis et al. 1992; Migliore et al. 1997; Jones et al. 2002; Pomati et al. 2004; Isidori et al. 2005) and one chronic 7-day study with *Ceriodaphnia dubia*. For *D. magna*, Isidori et al. (2005) reported a 24-h EC50 of 22.45 mg/L and for *C. dubia*, a 48-h EC50 of 10.23 mg/L, each based on immobilization. The 7-day EC50 for *C. dubia* was 0.22 mg/L based on population growth inhibition. Basing the effective concentration on mortality, Dojmi di Delupis et al. (1992) reported a 24 and 48-h EC50 of 388 mg/L and 211 mg/L, respectively, for *D. magna*.

Concentrations of DH and ET in pooled water sampled from test chambers of all treatment groups are presented in Table 2. Diphenhydramine hydrochloride and ET were not present in any water samples from the control treatment group.

Table 2 The diphenhydramine hydrochloride (DH) and erythromycin thiocyanate (ET) concentrations in pooled samples from test chambers where *Daphnia magna* were continuously exposed for 21 days

Treatment group (μg/L)	n	Mean measurement (μg/L)	SD (μg/L)
DH 0.12	22	0.120	0.040
DH 62	16	70.6	20
DH 620	14	846	76
ET 0.60	22	0.438	0.24
ET 300	22	248	90
ET 3000	22	2990	850
DH/ET 62/300	14	84.2/318	5.8/71

Only one *D. magna* in the control treatment group died and was found dead on day 13. Death was most likely associated with the injury incurred on day 12 while the analyst was removing young from the chamber.

No *D. magna* died in the 0.12-μg/L DH group. All *D. magna* in the 62-μg/L DH group were dead by Day 15 and all were dead by Day 14 in the 620-μg/L group. Exposure to DH explained a significant amount of the variability in the probability of death and the time to death (degrees of freedom, DF = 3; Log-Rank Chi-square = 35.7524, $p < 0.01$). Times to death for the control and 0.12-μg/L groups were not significantly different from each other, but were significantly longer than the times to death for the 62 and 620-μg/L DH groups (Table 3).

No *D. magna* died in the 0.60-μg/L ET group. Only one *D. magna* died in the 300-μg/L ET group. Six of seven *D. magna* in the 3,000-μg/L ET group were dead by Day 14; one survived through the trial. Exposure to ET explained a significant amount of the variability in the probability of death and the time to death (DF = 3; Log-Rank Chi-square = 20.1533, $p < 0.01$). The times to death for the control, 0.60, and 300-μg/L ET groups were not significantly different from each other, but were significantly longer than the times to death for the 3,000-μg/L ET group (Table 3).

All *D. magna* in the DH/ET mixture group were dead by study day 14. The time to death for the mixture group was significantly shorter than the time to death in the control group (DF = 1; Log-Rank Chi-square = 14.3846, $p < 0.01$). The time to death for the mixture group was not significantly different than the times to death for the 62- and 620-μg/L DH groups and the 3,000-μg/L ET group. Time to death was significantly shorter than the times to death for the 0.60-, and 300-μg/L ET groups (Table 3; DF = 2, Log-Rank Chi-Square = 18.6546, $p < 0.01$). Therefore, toxicity in this group was most likely due to DH.

The lengths of *D. magna* surviving to the end of the trial among all treatment groups were not significantly different. The mean lengths among the groups ranged from 4.42 to 4.60 mm.

The groups that produced young were the control, 0.12-μg/L DH, 0.60-, 300-, and 3,000-μg/L ET groups (Table 3). There was no significant difference in days to first brood among all groups where young were produced. The mean time to first brood was 9 days for all groups producing young.

The number of broods produced was inversely related to DH (DF = 3, Chi-Square = 500.78, $p < 0.01$) and ET concentrations (DF = 3, Chi-Square = 28.08, $p < 0.01$). The total numbers of broods produced in the control and 0.12-μg/L DH groups were not significantly different from each other but were significantly greater than the total numbers of broods produced in the 62-, 620-μg/L DH groups ($p < 0.01$). The total numbers of broods produced in the control, 0.60-, and 300-μg/L ET groups were not significantly different from each other, but were significantly greater than the number of broods produced in the 3,000-μg/L ET group ($p < 0.01$).

Table 3 Production and survival data from *Daphnia magna* continuously exposed to diphenhydramine hydrochloride (DH) and erythromycin thiocyanate (ET) for 21 days

Treatment group (μg/L)	Mean time to death (days)	Adults with broods	Young in first brood mean (range)	Broods per treatment group	Young per treatment group
0.0	19.9[a]	7	11[c] (3–25)	32[d]	1,071[e]
0.12 DH	>21[a]	7	10[c] (5–25)	35[d]	1,207[e]
62 DH	12.4[b]	0	–	–	–
620 DH	9.7[b]	0	–	–	–
0.60 ET	>21[a]	7	13[c] (3–23)	34[d]	1,328[e]
300 ET	18.3[a]	6	14[c] (4–25)	30[d]	1,100[e]
3000 ET	11.1[b]	4	10[c] (5–18)	9	247
62 DH/300 ET	11.0[b]	0	–	–	–

Data with the same letter were not significantly different ($p > 0.05$)

The total number of young produced was inversely related to DH concentration (DF = 3, Chi-Square = 237.53, $p < 0.01$) and ET (DF = 3, Chi-Square = 30.71, $p < 0.01$). The total numbers of young produced in the control and 0.12-μg/L DH groups were not significantly different from each other but were significantly greater than the total numbers of young produced in the 62-, and 620-μg/L DH groups ($p < 0.01$). Likewise, the total numbers of young produced in the control, 0.60-, and 300-μg/L ET groups were not significantly different from each other but were significantly greater than the number of young produced in the 3,000-μg/L ET group ($p < 0.01$).

In summary, continuous exposure to DH concentrations about 5 times greater than the maximum reported environmental concentration of 0.023 μg/L for 21 days did not significantly impact *D. magna* survival and production. Also, continuous exposure to ET concentrations about 40 times the maximum reported environmental concentration of 6 μg/L for 21 days did not significantly impact *D. magna* survival and production. Therefore, environmental concentrations of DH and ET most likely do not affect the survival, reproduction, and growth of chronically exposed *D. magna*.

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