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Acute toxicity of benzoic acids to the crustacean *Daphnia magna*

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Abstract

The acute immobilization toxicity of benzoic acids substituted with hydroxyl and/or methoxyl groups on the aromatic ring was determined for the freshwater crustacean *Daphnia magna* under neutralized condition (initial pH: 7.45 ± 0.05). Toxicity, expressed as EC_{50} value, varied depending largely on the number and position of phenolic hydroxyl groups. Especially, benzoic acids with *ortho*-substituted hydroxyl groups were more toxic than benzoic acids with *meta*- and/or *para*-substituted hydroxyl groups. Whereas the limited data indicated that methoxyl substitution had relatively small and variable effects on the toxicity. Of the tested compounds, 2,4,6-trihydroxybenzoic acid showed the highest toxicity with the 48 h EC_{50} of $10\ \mu mol\ l^{-1}$. This was 700 times as toxic as the parent benzoic acid (48 h $EC_{50} = 7.0\ mmol\ l^{-1}$) and about two orders of magnitude higher than those previously reported for monohalogenated benzoic acid derivatives in *Daphnia*. Within the subgroups based on the number of hydroxyl groups (N_{OH}), the toxicity variations due to the position of hydroxyl groups appeared to be correlated with the logarithms of *n*-octanol/water partition coefficients ($\log P_{ow}$). The toxicity of benzoic acids existing almost entirely as their ionized forms could be expressed as simple structure–toxicity relationships using these two descriptors (N_{OH} and $\log P_{ow}$).

Keywords: *Daphnia magna*; Toxicity; Benzoic acids; Hydroxyl group; $\log P_{ow}$

1. Introduction

Benzoic acid derivatives having a general structure C_6–C_1 are widely used as industrial chemicals, agrochemicals, pharmaceuticals and consumer products. On the other hand, a number of benzoic acid derivatives with variations including hydroxylation and methoxylation are known to be a group of secondary plant metabolites and also aerobic microbial degradation products of lignin, an important plant cell wall polymer (Chen and Chang, 1985). Some of these naturally occurring benzoic acid derivatives are shown to have various biological activities (Tomás-Barberán, 2000).

Furthermore, significant environmental sources of benzoic acids are the microbial metabolism of a variety of natural and anthropogenic aromatic compounds, since benzoic acid derivatives are very common structural units among the identified or estimated degradation products of aromatic compounds by aerobic microorganisms (Smith, 1990; Habe and Omori, 2003). However, toxic effects of stable degradation intermediates from aromatic compounds are not fully elucidated.

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Numerous studies have been made on the antibacterial, antifungal, and antiviral activities of natural and synthetic phenolic compounds including various substituted benzoic acids (Friedman et al., 2003, and references cited therein). However, studies on the effects of benzoic acids, as well as other carboxylic acids, to aquatic organisms have been limited (Fiorentino et al., 2003). Toxicity data for some halogenated benzoic acids in the bacteria, ciliate, daphnids, and fish have been reported (Zhao et al., 1996; Muccini et al., 1999). Whereas the toxicity of substituted phenols to *Daphnia* has been investigated by many researchers (Kopperman et al., 1974; LeBlanc, 1980; Devillers and Chambon, 1986; Devillers, 1988; Kühn et al., 1989; Jin et al., 1998; Abe et al., 2000).

The objective of this study was to establish data for acute toxicity of benzoic acids substituted with hydroxyl and/or methoxyl groups to the crustacean *Daphnia magna*. Furthermore, the data were compared with those of halobenzoic acids in the literature, and used to gain some information regarding the structure–toxicity relationships underlying the toxicity of these compounds. *D. magna*, an important freshwater invertebrate species in aquatic food webs, has been used world-wide for many years as a representative test species for ectoxicological evaluation of industrial chemicals (OECD, 1984).

2. Materials and methods

2.1. Organism and culture conditions

Daphnia magna obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan was used throughout this study. Neonatal daphnids were obtained from continuous cultures in 1 liter glass beakers at 21 ± 0.3 °C, in dechlorinated and conditioned tap water (total hardness, $\approx$100 mg l^{-1} as $CaCO_3$; pH 7.5 ± 0.1), 16 h light: 8 h dark photoperiod and a density of below 20 per beaker. The medium was renewed three times a weak and daphnids were fed daily with the green alga *Selenastrum capricornutum* NIES-35 ($3.0–3.5 \times 10^8$ cells l^{-1}), cultured also in our laboratory.

2.2. Chemicals and test solutions

Most chemicals (purity, minimum %) were used as purchased from the following companies: benzoic acid (99.5), 4-hydroxybenzoic acid (97), 2,3-dihydroxybenzoic acid (98), 2,5-dihydroxybenzoic acid (98), 4-hydroxy-3-methoxybenzoic acid (95), 4-methoxybenzoic acid (98), 3,4-dimethoxybenzoic acid (97), 3,4,5-trimethoxybenzoic acid (98), methyl 4-hydroxybenzoate (99), and 4-hydroxybenzaldehyde (98), from Wako Pure Chemical Industries, Osaka, Japan; 3-hydroxybenzoic acid (98), 2-hydroxybenzoic acid (99.5), 3,4-dihydroxybenzoic acid (98), and 3,4,5-trihydroxybenzoic acid (98) from Nacalai tesque, Kyoto, Japan; 2,4-dihydroxybenzoic acid (98), 2,6-dihydroxybenzoic acid (98), 3,5-dihydroxybenzoic acid (98), 2,3,4-trihydroxybenzoic acid (95), 2,4,6-trihydroxybenzoic acid (98), and 3-hydroxy-4-methoxybenzoic acid (98) from Tokyo Chemical Industries, Tokyo, Japan; and 4-hydroxy-3,5-dimethoxybenzoic acid (98) from Sigma, St Louis, MO, USA. 4-Hydroxybenzyl alcohol was prepared by the reduction of 4-hydroxybenzaldehyde with $NaBH_4$ in methanol at 0 °C in almost quantitative yield. Other reagents used for *Daphnia* medium were of the highest purity available and purchased from Wako.

Measured values of the logarithm of the *n*-octanol/water partition coefficient ($\log P_{ow}$) were obtained from on-line interactive demo version of SRC physical properties database (Syracuse Research Corporation Web site, http://esc.syrres.com). If a measured value was unavailable, the $\log P_{ow}$ was estimated using the online version of the CLOGP program (Daylight Chemical Information Systems Web site, http://www.daylight.com). The experimentally determined negative logarithms of the first dissociation constants (pK_a) were also obtained from the SRC database. If a measured value was unavailable, the pK_a value was estimated using the SPARC on-line calculator (http://ibmlc2.chem.uga.edu/sparc). Table 1 shows some physico-chemical properties of benzoic acids and related compounds tested in the present study.

To prepare the highest test solution, a test compound was dissolved in the aerated test medium and the pH was, where necessary, adjusted carefully with 1 mol l^{-1} NaOH solution to 7.45 ± 0.05, and then diluted to prepare a series of test solutions. Test medium used was "moderately hard water" prepared from deionized and distilled water (total hardness, $\approx$100 mg $CaCO_3$ l^{-1}; USEPA, 1993), and after aeration the pH was adjusted to 7.45 ± 0.05 with 1 mol l^{-1} HCl.

The solution pH decreased below 6 at concentrations over 1 mmol l^{-1} of benzoic acids when dissolved in the test medium employed here, and daphnids exposed to the solutions showed 100% immobility. Consequently, the toxic effects of benzoic acids were not related to the exposed concentrations alone, under the non-neutralized condition. It was further noted that the pH adjustment of some test solutions containing catechol and hydroquinone type structures such as 2,3-, 2,5-, and 3,4-dihydroxybenzoic acids, and 2,3,4-, and 3,4,5-trihydroxybenzoic acids resulted in slightly colored preparations.

The concentrations of the resultant solutions were checked before and after (48 h) exposure experiments by the UV spectra (400–200 nm) using a UV–visible spectrophotometer, UV mini 1240 (Shimadzu, Kyoto, Japan). No significant spectral changes in most test solutions were observed, indicating the stability of these