Effects of antibacterial agents, levofloxacin and clarithromycin, on aquatic organisms

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Abstract Contamination of surface waters by pharmaceutical chemicals is an emerging environmental problem. This study evaluated the toxic effects of the antibacterial agents levofloxacin (LVFX) and clarithromycin (CAM), which are widely used in Japan, on aquatic organisms. Ecotoxicity tests using a bacterium, alga and crustacean were conducted. Microtox test using a marine fluorescent bacterium showed that LVFX and CAM have no acute toxicity to the bacterium. From the results of the *Daphnia* immobilisation test, LVFX and CAM did not show acute toxicity to the crustacean. Meanwhile, an algal growth inhibition test revealed that LVFX and CAM have high toxicity to the microalga. The phytotoxicity of CAM was about 100-fold higher than that of LVFX from a comparison of EC50 (median effective concentration) value. From the *Daphnia* reproduction test, LVFX and CAM also showed chronic toxicity to the crustacean. Concentrations of LVFX and CAM in the aquatic environment were compared with PNEC (predicted no effect concentration) to evaluate the ecological risk. As a result, the ecological risk of LVFX is considered to be low, but that of CAM is higher, suggesting that CAM discharged into an aquatic environment after therapeutic use may affect organisms in the aquatic environment.

Keywords Antibacterial agent; clarithromycin; ecotoxicity; levofloxacin; risk

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Introduction

Contamination of surface waters by pharmaceutical chemicals has recently been recognised as an emerging environmental problem (Ternes, 1998; Daughton and Ternes, 1999; Dietrich *et al.*, 2002; Heberer, 2002). These contaminants enter the environment mainly via sewage effluent from human sources and agricultural run-off from animal husbandry operations. Some pharmaceuticals are not totally eliminated in sewage treatment plants (STPs) because conventional treatment technologies used in STPs do not appear to be designed to remove these specific compounds completely (Ternes, 1998; Carballa *et al.*, 2004). In fact, pharmaceuticals have been detected from sewage effluents and river waters receiving discharge from STPs (Hirsch *et al.*, 1999; Kolpin *et al.*, 2002; McArdell *et al.*, 2003; Tixier *et al.*, 2003; Ashton *et al.*, 2004). Since pharmaceuticals are designed to deliver biological effects, the potential adverse effects of these chemicals on non-target organisms are very important, and yet little is known about such effects (Webb, 2001).

This study evaluated the effects of the antibacterial agents levofloxacin (LVFX) and clarithromycin (CAM), which are widely used in Japan, on aquatic organisms. The effects of the antibacterial agents on a bacterium (*Vibrio fischeri*), an alga (*Pseudokirchneriella subcapitata*) and a crustacean (*Daphnia magna*) were examined. Additionally, ecological risk of LVFX and CAM was evaluated by comparison between concentrations in the aquatic environment and ecotoxicity results.

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Materials and methods

Test chemicals

The antibacterial agents LVFX and CAM were selected as the target chemicals to assess the ecotoxicity. These antibacterial agents are used widely in Japan for therapy of human beings. LVFX and CAM were purchased from Wako Chemical (purity >90%). The chemical structures of LVFX and CAM chemicals are shown in Figure 1.

Microtox test

Microtox reagent (*Vibrio fischeri*) and other required test solutions were purchased from Azur Environmental (Carlsbad, CA, USA). The experiment was carried out in accordance with the operation protocol of the Microtox acute toxicity test method. Luminescence was measured with a Microtox temperature-controlled photometer (Microtox Model 500, Azur Environmental) connected to a personal computer.

Algal growth inhibition test

This test is based on the measurement of growth inhibition of the alga *Selenastrum capricornutum*, recently renamed *Pseudokirchneriella subcapitata*, during 96 h of exposure. *P. subcapitata* (NIES-35) was obtained from culture collections in the National Institute for Environmental Studies, Japan. The tests were carried out using 96-well microplates according to the method of Blaise *et al.* (1986) and St-Laurent *et al.* (1992), with minor modifications. Initial cell densities were adjusted to 10^4 cells/mL. Algae were incubated at 24 °C under continuous white light (4,000 lux) and shaken automatically at 120 rpm. Absorbance (450 nm) was measured using a microplate reader (Wallac, ARVO SX-1420) during 96 h of incubation to monitor cell growth in microplate wells. Cell densities were obtained from a calibration curve of absorbance vs. cell density.

Daphnia acute immobilisation test

This test is based on the measurement of mobility inhibition of the crustacean *D. magna* during 48 h of exposure. Test animals were obtained from the National Institute for Environmental Studies, Japan. The test animals were maintained in reconstituted water, Elendt M-4 (Elendt, 1990) under 16:8 light:dark cycle at 20 °C with *Chlorella* sp. as food. An acute test with *D. magna* was conducted according to the OECD method (OECD, 1984). Twenty daphnids younger than 24 h were used for the controls and each treatment subdivided into four replicates each containing five daphnids. Culture volume was 50 mL. Immobility was observed after 24 and 48 h. Data after 48 h of exposure were used to calculate the effect.

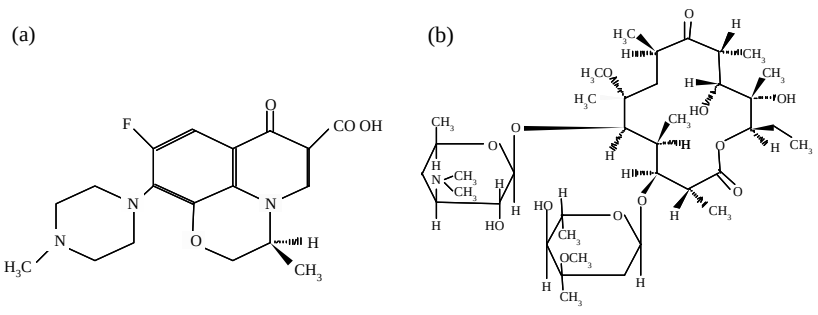

Figure 1 Chemical structures of (a) levofloxacin (LVFX) and (b) clarithromycin (CAM)

Daphnia reproduction test

This test is based on the measurement of reproduction inhibition of the crustacean *D. magna* during 21 d of exposure. Test animals were obtained from the National Institute for Environmental Studies, Japan. Test animals were maintained in reconstituted water, Elendt M-4 (14) under 16:8 light:dark cycle at 20 °C with *Chlorella* sp. as food. The reproduction test with *D. magna* was conducted according to the OECD method (OECD, 1998). Test solution (50 mL) and a test animal younger than 24 h were added into a beaker. Ten replicates were prepared for controls and each treatment and placed in an incubator maintained at 20 °C under 16:8 light:dark cycle. The test solution was renewed three times a week. The food for *D. magna* was *Chlorella* sp. and it was given six times a week. Its carbon level was 0.2 mg, as measured with a TOC meter (TOC-5000, Shimadzu Inc.), per animal a day. The chronic test lasted 21 d. If offspring were born, they were discarded after counting.

Statistical analysis

Test results were statistically analysed. EC50 (median effective concentration) values of LVFX and CAM were calculated from a concentration and inhibition curve. Dunnett's method was employed to compare the treatment with the control to determine LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration). Ecotox Statics ver.2.3 (The Japanese Society of Environmental Toxicology) was used to determine the LOEC and NOEC.

Results and discussion

Effects of levofloxacin and clarithromycin on bacterium

Microtox is an acute toxicity test using bacteria, detecting the phenomenon that toxic substances reduce the luminescence of marine bacteria (*Vibrio fischeri*). The results of the Microtox test after 15-min exposure to LVFX and CAM are presented in Figure 2. LVFX and CAM, which ranged from 0.032–8.2 mg/L, had little toxic effect on the test marine bacterium. At a maximum concentration of 8.2 mg/L, light emission released from luminescent bacterium was not reduced, which means that LVFX and CAM have little toxic effect at that concentration. Therefore, it is suggested that the antibacterial agents LVFX and CAM have little acute toxicity to bacteria. Meanwhile, Microtox is an acute toxicity test and the toxic effect of test chemicals to the growth of bacteria cannot be detected. Thus, the toxic effect of LVFX and CAM to the growth of bacteria will need to be studied.

Effects of levofloxacin and clarithromycin on alga

The algal growth inhibition test was conducted to evaluate the phytotoxicity of the antibacterial agents LVFX and CAM. The phytotoxicity of LVFX and CAM to the microalga (*P. subcapitata*) after 96 h exposure is shown in Figure 3. The toxic effect of

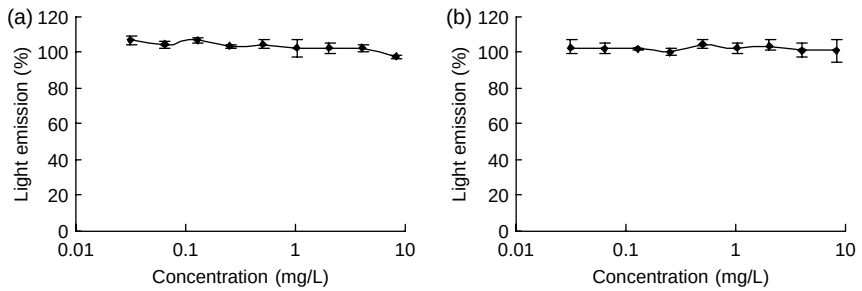

Figure 2 Effects of (a) levofloxacin and (b) clarithromycin on bacterium (*Vibrio fischeri*)

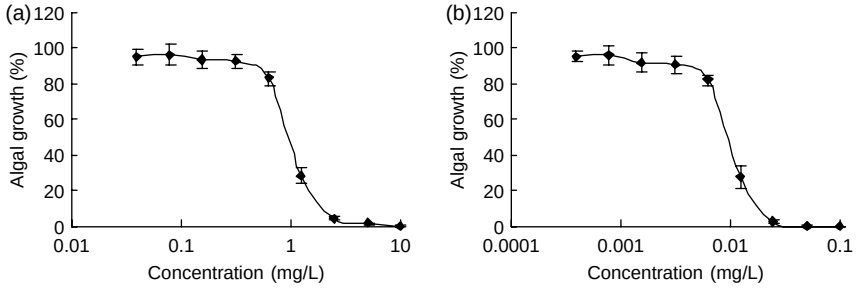

Figure 3 Effects of (a) levofloxacin and (b) clarithromycin on alga (*Pseudokirchneriella subcapitata*)

LVFX to the microalga was observed at concentrations above 630 μg/L. The growth of the microalga was not observed at concentrations exceeding 2,500 μg/L. The EC50 value calculated from a concentration and inhibition curve was 1,200 μg/L. LOEC and NOEC determined with Dunnett's method were 630 and 310 μg/L, respectively. The phytotoxicity of CAM to the microalga (*P. subcapitata*) after 96 h exposure is also shown in Figure 3. The toxic effect of CAM to the microalga was observed at concentrations above 6.3 μg/L. Growth of the microalga was never observed at concentrations exceeding 25 μg/L. The EC50 value calculated from a concentration and inhibition curve was 11 μg/L. LOEC and NOEC were 6.3 and 3.1 μg/L, respectively.

From the results of the algal growth inhibition test, the toxicity of CAM on the microalga was much higher than that of LVFX. Comparison of EC50 values between LVFX and CAM showed that the toxicity of CAM was about 100-fold higher than that of LVFX. One possible explanation for this difference in toxicity is hydrophobicity of the chemicals. It was reported that toxicity of chemicals is related to the hydrophobicity (Deneer *et al.*, 1989; Blum and Speece, 1990; Ikemoto *et al.*, 1992). The values of log K_{ow}, which is considered to be an indicator of the hydrophobicity for chemicals, for LVFX and CAM are reported to be 0.553 and 7.18, respectively. The difference in log K_{ow} values might relate to the difference in the toxicities of LVFX and CAM on microalgae.

Effects of levofloxacin and clarithromycin on crustacean

The *Daphnia* acute immobilization test was conducted to assess the toxic effects of the antibacterial agents LVFX and CAM on the crustacean *D. magna*. The end point of this test was mobility of the *Daphnia*. The results of the toxicity test using the crustacean after 48 h exposure are shown in Figure 4. Toxic effects were not observed in comparing the treatment with the control at concentrations ranging from 0.01 to 10 mg/L. LVFX and CAM did not show any acute toxicity to the crustacean *D. magna*.

The *Daphnia* reproduction test was also conducted to assess the chronic toxic effects of LVFX and CAM on the crustacean *D. magna*. The end point of this test was offspring

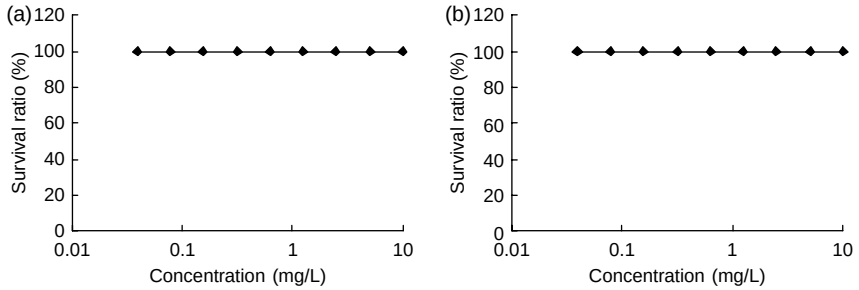

Figure 4 Effects of (a) levofloxacin and (b) clarithromycin on immobilization of crustacean (*Daphnia magna*)

by the parent animal. Figure 5 shows the temporal change of offspring by the parent animal. Offspring started after the 10th day for reconstituted M4 water, which is the control, and continued to the end of the experiment. The number of offspring was approximately 12–30 animals per parent a day. The total number of offspring during the experimental period (21 days) was approximately 160 animals per parent for the control. Meanwhile, under exposure to LVFX at the concentration of 0.5 mg/L, the number of offspring was reduced to approximately 5–17 animals per parent a day. The total number of offspring in the case of treatment of LVFX during the experimental period (21 days) was reduced to approximately 70 animals per parent.

The results of the *Daphnia* reproduction test of LVFX and CAM for the crustacean are shown in Figure 6, which shows the relationship between concentrations of antibacterial agents and offspring per parent animal. The toxic effect of LVFX on the reproduction of the crustacean was observed at concentrations above 63 μg/L. Offspring by the parent animal were hardly observed at a concentration of 1,000 μg/L. The EC50 value calculated from a concentration and inhibition curve was 340 μg/L. LOEC and NOEC determined with Dunnett's method were 63 and 31 μg/L, respectively. The chronic toxicity of CAM to the crustacean after 21 days of exposure is also shown in Figure 6. The toxic effect of CAM on the crustacean was observed at concentrations above 6.3 μg/L. The EC50 value calculated from a concentration and inhibition curve was 40 μg/L. LOEC and NOEC determined with Dunnett's method were 6.3 and 3.1 μg/L, respectively.

From the results of the *Daphnia* reproduction test, the chronic toxicity of CAM on the crustacean was much higher than that of LVFX, which is a similar trend to the results of the algal growth inhibition test. Comparison of the EC50 value between LVFX and CAM showed that the toxicity of CAM to *Daphnia* reproduction was approximately 10-fold higher than that of LVFX. The toxicity of CAM to the microalga was approximately 100-fold higher than that of LVFX. Thus, the difference of toxicity between LVFX and

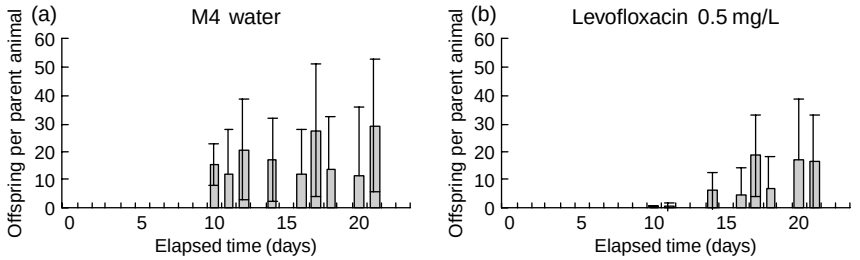

Figure 5 Offspring of crustacean *Daphnia magna*

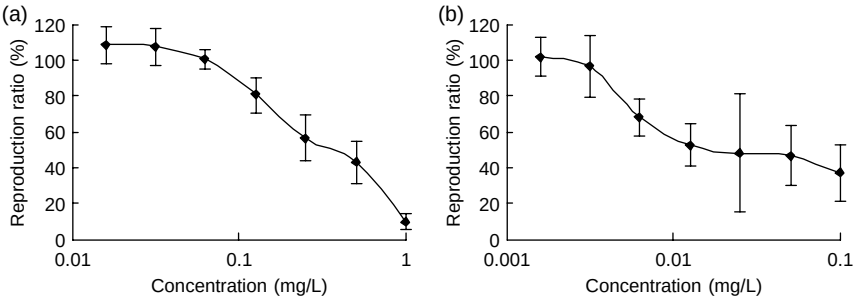

Figure 6 Effects of (a) levofloxacin and (b) clarithromycin on reproduction of crustacean (*Daphnia magna*)

CAM to *Daphnia* reproduction was smaller. However, these results indicate that CAM is much more toxic to aquatic organisms, such as algae and crustaceans, than LVFX.

First approach of risk evaluation for levofloxacin and clarithromycin

It is important to assess the toxic effects of LVFX and CAM on aquatic organisms. Ecological risk was evaluated by comparison between concentrations in the aquatic environment and ecotoxicity results obtained in this study.

A survey of LVFX and CAM was conducted for the secondary effluent from STPs in Japan (Yasojima *et al.*, 2004). Conventional activated sludge process is applied in these STPs. LVFX was detected at concentrations ranging from 152 to 323 ng/L. CAM ranged from 303 to 567 ng/L (Figure 7).

LVFX and CAM did not show acute toxicity from the results of the Microtox test and *Daphnia* acute immobilisation test, but did show toxicity based on the results of the algal growth inhibition test and *Daphnia* reproduction test. EC50, LOEC and NOEC obtained from the algal growth inhibition test were 1,200, 630 and 310 μg/L for LVFX and 11, 6.3 and 3.1 μg/L for CAM, respectively. EC50, LOEC and NOEC obtained from the *Daphnia* reproduction test were 340, 63 and 31 μg/L for LVFX and 40, 6.3 and 3.1 μg/L for CAM, respectively. From these results, the most sensitive values to evaluate the ecotoxicity of LVFX and CAM were summarised as shown in Table 1. Results of the *Daphnia* reproduction test were adopted for the ecotoxicity of LVFX, and the results of the algal growth inhibition test were adopted for the ecotoxicity of CAM. Considering 100 as a safety factor, PNEC (predicted no effect concentration) calculated from NOEC resulted in 310 ng/L for LVFX and 31 ng/L for CAM.

Concentrations of LVFX and CAM in the aquatic environment were compared with PNEC to evaluate the ecological risk. Risk ratio was calculated from the concentration divided by the PNEC. Figure 8 shows the results of the risk ratio. As for LVFX, the risk ratio was nearly 1 or lower and so the ecological risk is considered to be low. On the other hand, for CAM, risk ratio ranged from 9.8 to 18, and exceeded 1 for all the STP samples. Therefore, CAM poses an ecological risk when discharge from STPs is not diluted sufficiently, and it is suggested that CAM discharged into an aquatic environment after therapeutic use may affect organisms in the aquatic environment.

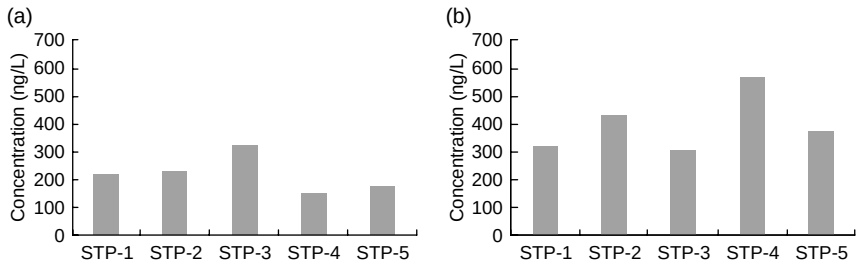

Figure 7 Concentrations of (a) levofloxacin and (b) clarithromycin in secondary effluent of sewage treatment plants (Yasojima *et al.*, 2004)

Table 1 EC50, LOEC and NOEC of levofloxacin (LVFX) and clarithromycin (CAM)

Substance	EC50 (μg/L)	LOEC (μg/L)	NOEC (μg/L)
Levofloxacin	340[a]	63[a]	31[a]
Clarithromycin	11[b]	6.3[b]	3.1[b]

[a]Result obtained from *Daphnia* reproduction test
[b]Result obtained from algal growth inhibition test

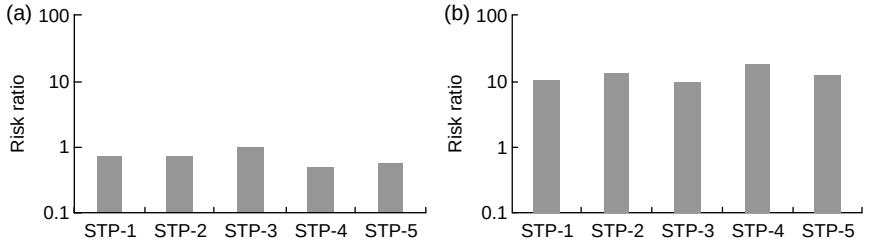

Figure 8 Risk ratios of (a) levofloxacin and (b) clarithromycin

Conclusions

This study evaluated the toxic effects of antibacterial agents levofloxacin and clarithromycin, which are widely used in Japan, on aquatic organisms. Ecotoxicity tests using a bacterium, alga and crustacean were conducted. Microtox test using a marine fluorescent bacterium showed that LVFX and CAM have no acute toxicity to the bacterium. From the results of the *Daphnia* immobilisation test, LVFX and CAM did not show acute toxicity to the crustacean. Meanwhile, the algal growth inhibition test revealed that LVFX and CAM have high toxicity to the microalga. The phytotoxicity of CAM was about 100-fold higher than that of LVFX from a comparison of EC50 values. LVFX and CAM also showed chronic toxicity to the crustacean from the *Daphnia* reproduction test. Concentrations of LVFX and CAM in the aquatic environment were compared with PNEC to evaluate the ecological risk. As a result, the ecological risk of LVFX is considered to be low, but that of CAM is higher, suggesting that CAM discharged into an aquatic environment after therapeutic use may affect organisms in the aquatic environment.

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