Original Article

www.ToxicolRes.org
Toxicological Research

Toxicol. Res. Vol. 35, No. 2, pp. 155-160 (2019)
https://doi.org/10.5487/TR.2019.35.2.155

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Effect of Combined Exposure to EDTA and Zinc Pyrithione on Pyrithione Absorption in Rats

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Abstract

Zinc pyrithione (ZnPT) is a coordination complex of zinc and has been used widely as an anti-dandruff agent in shampoos. Many shampoos contain both ZnPT and EDTA, a chelating agent speculated to increase ZnPT absorption, thereby raising concerns about neurotoxicity. Here, we investigated the effect of EDTA on ZnPT absorption by direct comparison of ZnPT and pyrithione (PT) concentrations in shampoo formulations, and by pharmacokinetic analysis of ZnPT, PT, and 2-methanesulfonylpyridine (MSP), the main ZnPT metabolite, in rat plasma or urine following exposure to shampoo containing ZnPT alone or a combination of ZnPT and EDTA. Approximately 17.3% of ZnPT was converted to PT by the addition of EDTA in the shampoo formulation. Plasma ZnPT and PT concentrations were not measured up to 24 hr after treatment with shampoo containing 1% ZnPT or 1% ZnPT + 2% EDTA in all rats. However, PT amount in 24-hr urine sample, MSP concentration in plasma, and MSP amount in 24-hr urine sample were approximately 4-, 2.6-, and 2.7-fold higher, respectively, in the 1% ZnPT + 2% EDTA shampoo group than in the 1% ZnPT shampoo group. As confirmed by the formulation analysis and *in vivo* pharmacokinetic analysis, the exposure of ZnPT could be increased by the absorption of PT due to partial dissociation of ZnPT into PT.

Key words: Zinc pyrithione, Pyrithione, Pharmacokinetic, Shampoo

INTRODUCTION

Zinc pyrithione (ZnPT) is a coordination complex of zinc (Fig. 1), and its use has been widely allowed in the European Union as an anti-dandruff agent in hair dressing formulations and shampoos, as a preservative at a concentration of 0.5% in cosmetic rinse-off hair-care products, and at a concentration of 1% in rinse-off antidandruff hair-care products (1). It has also been intensively used worldwide as an antifouling agent in painting formulations.

The safety of ZnPT or pyrithione (PT) derivatives, such as sodium pyrithione (NaPT) and copper pyrithione (CuPT), has been studied in several animal species through different routes of administration. Dogs showed ocular damage

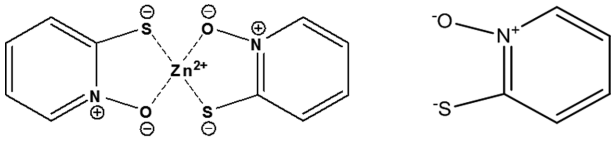

Fig. 1. Structure of zinc pyrithione (A) and pyrithione (B).

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Abbreviations: AUC, area under the concentration-time curve; CuPT, copper pyrithione; d3-AGM130, d_3-5-Nitro-5′-hydroxy-indirubin-3′-oxime; C_{max}, maximum plasma concentration values; MSP, 2-methanesulfonylpyridine; MRM, multiple reaction monitoring; PT, pyrithione; NaPT, sodium pyrithione; T_{max}, the time at maximum plasma concentration values; ZnPT, Zinc pyrithione.

pISSN: 1976-8257 eISSN: 2234-2753

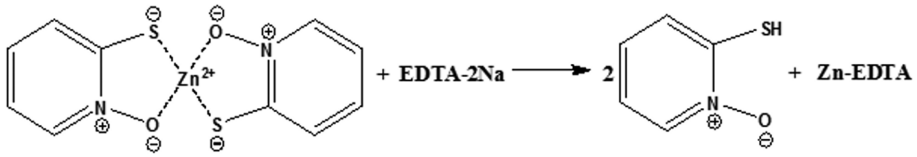

Fig. 2. Reaction of ZnPT and EDTA.

involving the tapetum lucidum after treatment with oral ZnPT at 6-12 mg/kg/day for 6 days (2,3). Rats and rabbits showed hindlimb weakness or paralysis after subchronic and chronic administration of ZnPT (3,4). ZnPT can transform into CuPT by transchelation with copper ion in both laboratory and natural conditions (5,6). The environmental toxicity of ZnPT or CuPT and their degradation products on microalgae, macrophytes, crustaceans, fish, sea urchin, and other organisms have been studied (7-17). More than 200 shampoos contain both ZnPT and EDTA, a chelating agent. EDTA is useful to improve stability in shampoo formulations. ZnPT has been reported to show low penetration, whereas NaPT, with high water solubility, was shown to be absorbed through the skin in much greater amount than ZnPT (18). Thus, NaPT was prohibited as an ingredient in shampoo formulations. However, the current situation has been left without any criteria with respect to the formulation of ZnPT and EDTA at home and abroad.

These compositions are speculated to increase ZnPT absorption by EDTA, and the resulting toxicity is concerning (Fig. 2). ZnPT is absorbed in small quantities through the skin and is rapidly metabolized into its metabolites. Among the metabolites, 2-methanesulfonylpyridine (MSP) has been identified as a major serum metabolite of ZnPT (19). Thus, in this study, we evaluated the effect of EDTA on systemic ZnPT exposure by measuring ZnPT, PT, and MSP contents in rat plasma and urine following treatment with shampoos containing ZnPT alone or a combination of ZnPT and EDTA.

MATERIALS AND METHODS

Chemicals. ZnPT, PT, MSP, EDTA, imipramine HCl (an internal standard for MSP analysis), and d_3-5-Nitro-5′-hydroxy-indirubin-3′-oxime (d3-AGM130; an internal standard for ZnPT and PT analyses) were purchased from Sigma-Aldrich Chemical Corporation (Milwaukee, WI, USA). Water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade and used without further purification.

Preparation of shampoo formulations. Shampoo formulations were prepared by adding ZnPT alone or ZnPT and EDTA in a commercial shampoo product (Head & Shoulder, Cincinnati, OH, USA) to yield a final concentration of 5% ZnPT or 5% ZnPT + 10% EDTA, respectively. The shampoo formulations were mixed thoroughly, then diluted 5 times with distilled water. The final diluted shampoo formulations (1% ZnPT or 1% ZnPT + 2% EDTA) were used in experiments.

Animal experiments. Male Sprague-Dawley rats (approximately 8 weeks old, n = 12) (Central Lab. Animal Inc., Seoul, Korea) were used for experiments. The rats were kept under controlled conditions (ambient temperature of 23 ± 2°C, humidity of 50 ± 10%, 12-hr light/dark cycle). Food (Central Lab. Animal Inc.) and water were supplied *ad libitum*. The rats were acclimated to the laboratory conditions for 7 days. All experimental procedures were approved by Dankook University's Institutional Animal Care and Use Committee (DUIACUC), which adheres to the guidelines issued by the Institution of Laboratory of Animal Resources (ILAR). The rats were fasted for at least 12 hr prior to the start of experiment. Each group consisted of 6 males. The animals were approximately 10 weeks old at the study date. Individual body weights ranged from 270 to 350 g. The animals were then assigned to two groups. Group 1 was treated with shampoo containing 1% ZnPT, whereas group 2 was treated with shampoo containing 1% ZnPT + 2% EDTA. The rats were anesthetized with zoletil 50 (5 mg/kg) administered intramuscularly, and then cannulated using a polyethylene tube into the jugular vein. The distal end of the cannula was then tunneled subcutaneously to the back of the neck, where it was exteriorized. Blood coagulation in the cannula was prevented through introduction of 0.2 mL of heparinized normal saline (20 units/mL). After all cannulated rats completely recovered from anesthetization, they were partially anesthetized with zoletil 50 (3 mg/kg) administered intramuscularly, and exposed to shampoo solutions. Six cannulated rats of each group were immersed below the cannulated part in each shampoo solution by holding for 10 sec, dried by wiping, and then placed in metabolic cages. Blood samples were collected from the cannulated rats via the jugular vein before shampoo treatment and at 0.5, 1, 2, 3, 4, 6, 8, and 24 hr after shampoo treatment. Approximately 0.3 mL of blood samples were collected using heparin-coated disposable syringes. Immediately after collection, blood samples were centrifuged for 1-2 min at 10,000-13,000 rpm. The obtained

plasma samples were stored at −70°C until analysis. Urine samples were collected for 0-24 hr. After urine collection at 24 hr, the cages were washed with 10 mL distilled water, and the washings were mixed into the 24-hr urine sample. The final urine volume of each animal was reported, and each 1 mL of urine sample was stored at −70°C until analysis.

Determination of ZnPT and PT in rat plasma and urine. Chloroform:MeOH (2:1, v/v) (150 μL) was added to plasma and urine samples (50 μL), and the mixtures were mixed thoroughly for 30 sec. After centrifugation at 4°C and 13,000 rpm for 5 min, the chloroform layers (50 μL) were diluted with MeOH (50 μL). The processed samples (5 μL) were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). An Agilent 1260 (Santa Clara, CA, USA) HPLC system coupled to an API4000 Q-trap (AB Sciex, Framingham, MA, USA) quadrupole tandem mass spectrometer was used to determine ZnPT and PT concentrations in plasma and urine. The HPLC column was a Capcell PAK MG (3 mm × 35 mm, 3 μm particle size) connected with a guard column (SecurityGuard C18 4 × 2 mm, 3 μm; Phenomenex, CA, USA). The separation was carried out with a mobile phase consisting of MeOH (0.1% formic acid)/20 mM ammonium acetate (90/10, v/v), with the following conditions: flow rate, 350 μL/min; injection volume, 5 μL; column oven temperature, 40°C; run time, 1.5 min. The Q-Trap mass spectrometer was operated in the positive ion mode using an electrospray ionization source. High-purity nitrogen gas was used for the nebulizer and curtain gases. The source temperature was set at 400°C with a curtain gas flow of 50 L/min. The ion spray voltage was set at 5,500 V and the collision energy were 20 and 15 V for ZnPT and PT, respectively. The following multiple reaction monitoring (MRM) transitions of the respective $[M+H]^+$ ions were used to quantify ZnPT and PT. ZnPT: *m/z* 316.9 → 190.0; PT: *m/z* 128.0 → 110.0; and d_3-AGM-130 (IS): *m/z* 342.0 → 279.0. Calibration curves exhibited excellent linearity over a range of 50-2,000 ng/mL for ZnPT and of 50 or 100-2,000 ng/mL for PT in rat plasma and urine (Supplementary Fig. 1, 2). The intra- and inter-accuracy values (%) were in a range of 98.3 to 110.5% for ZnPT and of 95.5 to 112.9% for PT, whereas the intra- and inter-precision values (CV, %) were less than 15% for ZnPT and PT in rat urine (Supplementary Table 1, 2).

Determination of MSP in rat plasma and urine samples. Internal standard solution (120 μL; imipramine 10 ng/mL in acetonitrile) was added to plasma and urine samples (30 μL), and the mixtures were vortex-mixed thoroughly for 30 sec. After centrifugation at 4°C and 13,000 rpm for 10 min, the supernatants were diluted with 2 times volume of distilled water. Each aliquot (100 μL) was transferred to autosampler vials and analyzed by LC-MS/MS. An Agilent 1260 (Agilent) HPLC system coupled to an API4000 (AB Sciex) quadrupole tandem mass spectrometer was used for sample analysis. The HPLC column was a Capcell PAK MG (2 mm × 75 mm, 3 μm particle size) connected with a guard column (SecurityGuard C18 4 mm × 2 mm; Phenomenex). The separation was carried out with a mobile phase consisting of acetonitrile/10 mM ammonium formate, pH 3.0 (50/50, v/v), with the following conditions: flow rate, 300 μL/min; injection volume, 5 μL; column oven temperature, 40°C; run time, 2.0 min. The electrospray ionization mass spectrometer was operated in the positive ion mode. The source temperature was set at 500°C. The ion spray voltage was set at 5,000 V and the collision energy was 35 V for MSP, respectively. The following MRM transitions of the respective $[M+H]^+$ ions were used to quantify MSP: *m/z* 158.2 → 78.0 and imipramine (IS): *m/z* 281.2 → 86.2. Calibration curves exhibited excellent linearity over a range of 5-1,000 ng/mL in rat plasma (Supplementary Fig. 3) and of 20-1,000 ng/mL in rat urine. The intra- and inter-accuracy values (%) in rat plasma were in a range of 97.2% to 99.9%, whereas the intra- and inter-precision values (CV, %) were less than 10% (Supplementary Table 3).

Data analysis. All chromatographic peaks were reviewed, and any integration corrections were made manually when necessary. Calibration curves were prepared using a linear regression with 1/x or $1/x^2$ weighting. All standard and sample concentrations were determined using internal standard areas versus analyte areas. Individual plasma concentration-time data of MSP were analyzed using non-compartmental method of the WinNonlin 2.1 pharmacokinetic program. Nominal sample times were used. The area under the concentration-time curve (AUC) was calculated using the linear log-linear trapezoidal rule. The maximum plasma concentration (C_{max}) values and the time at which it was observed (T_{max}) were directly determined from the plasma concentration-time profiles. All data were expressed as mean ± SD.

RESULTS

Analysis of shampoo formulations. ZnPT and PT concentrations in the shampoo formulations prepared in laboratory (Supplementary Fig. 4, Supplementary Table 4) were analyzed by LC-MS/MS, and the results are presented in Table 1. In the shampoo containing 1% ZnPT

Table 1. ZnPT and PT concentrations (mg/mL) in the shampoo solutions

Compound	1% ZnPT	1% ZnPT + 2% EDTA
ZnPT (mg/mL)	9.79	5.82
PT (mg/mL)	0.001	1.73

Table 2. ZnPT and PT concentrations in rat plasma and urine after shampoo treatment

Group	Plasma (ng/mL) n = 5		Urine (μg, mean ± SD) n = 5	
	ZnPT	PT	ZnPT	PT
1% ZnPT	BLQ	BLQ	1.0 ± 0.2	4.7 ± 1.7
1% ZnPT + 2% EDTA	BLQ	BLQ	1.0 ± 0.1	18.6 ± 11.5

BLQ: Below the lower limit of quantitation (50 ng/mL).

alone, ZnPT and PT concentrations were 9.79 and 0.001 mg/mL, respectively, whereas in that containing 1% ZnPT + 2% EDTA, ZnPT and PT concentrations were 5.82 and 1.73 mg/mL, respectively. Recovery rates (%) of ZnPT in shampoo containing 1% ZnPT or 1% ZnPT + 2% EDTA were 97.9 % and 58.2 %, respectively. Moreover, approximately 17.3% of ZnPT was converted into PT in the shampoo containing 1% ZnPT + 2% EDTA (Table 1).

ZnPT and PT in rat plasma and urine. Analytical methods of ZnPT and PT concentrations in rat plasma and urine were established and validated (Supplementary Fig. 1, 2, Supplementary Table 1, 2). Two rats, 1 from each group, died during the sampling procedure after catheterization. Therefore, samples from five rats of each group were analyzed. ZnPT and PT concentrations in rat plasma and 24-hr urine sample after treatment with shampoo containing 1% ZnPT or 1% ZnPT + 2% EDTA are shown in Table 2. Plasma ZnPT and PT concentrations were not measured up to 24 hr after treatment with shampoo containing 1% ZnPT or 1% ZnPT + 2% EDTA in all rats. ZnPT amount was measurable in the 24-hr urine sample of most rats of the 1% ZnPT + 2% EDTA shampoo group. However, there was no significant difference in urine ZnPT amount between the two groups, and most of urine ZnPT must have originated from the washing water of metabolic cages, which were contaminated with ZnPT from wiped rat hair. PT amount in 24-hr urine was approximately 4-fold higher in the 1% ZnPT + 2% EDTA shampoo group than in the 1% ZnPT shampoo group.

Exposure of MSP in rat plasma and urine. Analytical methods of MSP concentration in rat plasma and urine were established and validated (Supplementary Fig. 3, Supplementary Table 3). MSP concentration in rat plasma and MSP amount in rat 24-hr urine sample after treatment with shampoo containing 1% ZnPT or 1% ZnPT + 2% EDTA are presented in Table 3 and 5, respectively. Plasma MSP concentrations were not measurable up to 2 hr after treatment with shampoo containing 1% ZnPT, and up to 0.5 or 1 hr after treatment with shampoo containing 1% ZnPT + 2% EDTA. Thereafter, MSP concentration was gradually increased up to 24 hr in both groups. The pharmacokinetic parameters calculated from individual plasma MSP concentration in rats are presented in Table 4. The mean C_{max} and AUC_{last} values were 2.1- and 2.6-fold higher, respectively, in the 1% ZnPT + 2% EDTA group than in the 1% ZnPT group. MSP amount was measurable in the 24-hr urine sample of all rats of the 1% ZnPT shampoo and 1% ZnPT + 2% EDTA shampoo groups. MSP amount in 24-hr urine sample was approximately 2.7-fold higher in the 1% ZnPT + 2% EDTA shampoo group than in the 1% ZnPT shampoo group.

Table 3. Plasma MSP concentrations (ng/mL, mean ± SD) in rats after treatment with shampoo containing 1% ZnPT or 1% ZnPT + 2% EDTA

Time (hr)	1% ZnPT (n = 5)	1% ZnPT + 2% EDTA (n = 5)
Blank	BLQ	BLQ
0.5	BLQ	BLQ
1	BLQ	9.5 ± 10.3
2	BLQ	38.5 ± 26.8
3	7.6 ± 1.8	91.3 ± 51.7
4	11.7 ± 2.6	138 ± 68.1
6	23.4 ± 5.5	225 ± 100
8	35.9 ± 12.9	286 ± 122
24	565 ± 435	1175 ± 269

BLQ: Below the lower limit of quantitation (5 ng/mL).

Table 4. Pharmacokinetic parameters of MSP in rat plasma after treatment with shampoo containing 1% ZnPT or 1% ZnPT + 2% EDTA

Parameters	1% ZnPT	1% ZnPT + 2% EDTA
C_{max} (μg/mL)	0.57 ± 0.44	1.18 ± 0.27
AUC_{last} (μg*hr/mL)	4.9 ± 3.4	12.8 ± 1.4

Data are presented as mean ± SD.

Table 5. MSP amount (μg) in rat urine at 24 hr after shampoo treatment

Group	Urine (μg, mean ± SD)
	MSP
1% ZnPT (n = 5)	1.8 ± 1.2
1% ZnPT + 2% EDTA (n = 5)	4.9 ± 1.2

DISCUSSION

Zinc pyrithione (ZnPT) has been widely used as an antidandruff agent in hair dressing formulations and shampoos, and as an antifouling agent in painting formulations. The toxicity of ZnPT after oral, dermal, and inhalation administration has been examined in repeated toxicity studies using several animal species. A NOAEL of 500 μg/kg per day obtained from a chronic oral study (SCCS 2014) of ZnPT based on paralysis and hindlimb weakness, has been derived. The percutaneous absorption of ZnPT through the skin is very low with approximately 0.03-3.4%, compared with that of NaPT. Compared with ZnPT, NaPT showed much higher water solubility and is absorbed through the skin in much greater amounts (18). Therefore, NaPT was prohibited as an ingredient in shampoo formulations.

EDTA, a chelating agent, has been used as a component in shampoo formulations to improve stability. We investigated the effect of EDTA on ZnPT absorption in shampoo formulations. PT and MSP, among ZnPT metabolites (19,20), are used as indicators of systemic ZnPT exposure. In this study, a direct LC-MS/MS method was developed for quantitative determination of ZnPT, PT, and MSP concentrations in rat plasma and urine. Analytical results showed significant differences in ZnPT and PT concentrations between the two shampoo formulations tested in this study. Compared to those in the shampoo formulation without EDTA, ZnPT concentration decreased by approximately 59%, whereas PT concentration increased in the shampoo formulation with EDTA. These results indicated that ZnPT was partially dissociated to PT ion by chelating zinc ion of EDTA.

Plasma ZnPT concentrations were below the LLOQ in all samples from the 1% ZnPT and 1% ZnPT + 2% EDTA shampoo groups. Furthermore, there were no differences in PT concentration in plasma samples and ZnPT amount in 24-hr urine samples between the 1% ZnPT and 1% ZnPT + 2% EDTA shampoo groups. However, PT amount in 24-hr urine sample was approximately 4-fold higher in the 1% ZnPT + 2% EDTA shampoo group than in the 1% ZnPT shampoo group. After oral dosing, ZnPT is extensively absorbed by the gastrointestinal tract and rapidly eliminated via urine in rabbits, rats, monkeys, and dogs. The biotransformation of ZnPT is similar in all four of these species, and its major urinary metabolites are 2-pyridinethiol-1-oxide-S-glucoside, 2-pyridinethiol-S-glucuronide, and 2-pyridinethiol-1-oxide-S-glucuronide (20). Serum metabolites of ZnPT have been reported in rabbits, rats, monkeys, and dogs after oral dosing. Apparently, three minor metabolites, 2-(methylthio)pyridine-1-oxide, 2-(methylthio)pyridine, and 2-(methylsulfinyl) pyridine-1-oxide, are the intermediate compounds in the biotransformation of ZnPT to MSP (19). MSP has been identified as a major serum metabolite of ZnPT. Because MSP is a very stable and abundant metabolite of ZnPT, and positioned at the final step of the metabolic pathway of ZnPT, it can be used as a good indicator of systemic ZnPT exposure. In both groups, plasma MSP concentration gradually increased with time and reached a maximum concentration at 24 hr, the last time of blood collection. There were significant differences in MSP concentration between the two groups. MSP concentration, in terms of mean AUC_{last}, was approximately 2.6-fold higher in the 1% ZnPT + 2% EDTA shampoo group than in the 1% ZnPT shampoo group. Gibson *et al*. (19) reported that MSP in the circulation increased with time after ZnPT dosing in rabbits, rats, and monkeys, and it was the only prominent circulating metabolite at the later time points. Similar to the results in plasma, MSP amount in 24-hr urine sample was also approximately 2.7-fold higher in the 1% ZnPT + 2% EDTA shampoo group than in the 1% ZnPT shampoo group. As confirmed by the formulation analysis, this could be caused by increased absorption of PT due to partial dissociation of ZnPT into PT by EDTA. Systemic exposure to ZnPT could be increased by EDTA in cosmetic rinse-off hair-care products or antidandruff hair-care products, and chronic use of these products may result in an unexpected neurotoxicity. The current situation has been left without any criteria with respect to ZnPT and EDTA formulations at home and abroad. The effect of EDTA on systemic exposure to ZnPT should be confirmed through further studies, such as stability test of ZnPT in shampoos containing EDTA or patch test. However, rinse-off hair care products containing 1% ZnPT and EDTA are acceptable for safe use, according to risk assessment of ZnPT products including EDTA performed by the Ministry of Food and Drug Safety of Korea (MFDS).

ACKNOWLEDGMENTS

This work was supported by a grant (14172MFDS975) from the Ministry of Food and Drug Safety, Korea, in 2014.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Received February 18, 2019; Revised March 9, 2019; Accepted March 10, 2019

REFERENCES

1. Scientific Committee on Consumer Safety (SCCS) (2014) Opinion on Zinc Pyrithione (Colipa P81).
2. Cloyd, G.G., Wyman, M., Shadduck, J.A., Winrow, M.J. and Johnson, G.R. (1978) Ocular toxicity studies with zinc pyri-

dinethione. *Toxicol. Appl. Pharmacol.*, **45**, 771-782.
3. Snyder, F.H., Buehler, E.V. and Winek, C.L. (1965) Safety evaluation of zinc 2-pyridinethiol 1-oxide in a shampoo formulation. *Toxicol. Appl. Pharmacol.*, **7**, 425-437.
4. Sahenk, Z. and Mendell, J.R. (1977) Studies on the dying-back process of peripheral nerves using bis(N-oxopyridine-2-thionato)zinc(II). *Neurology*, **27**, 393.
5. Grunnet, K.S. and Dahllof, I. (2005) Environmental fate of the antifouling compound zinc pyrithione in seawater. *Environ. Toxicol. Chem.*, **24**, 3001-3006.
6. Thomas, K.V. (1999) Determination of the antifouling agent zinc pyrithione in water samples by copper chelate formation and high-performance liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry. *J. Chromatogr. A*, **833**, 105-109.
7. Bao, V.W., Leung, K.M., Kwok, K.W., Zhang, A.Q. and Lui, G.C. (2008) Synergistic toxic effects of zinc pyrithione and copper to three marine species: Implications on setting appropriate water quality criteria. *Mar. Pollut. Bull.*, **57**, 616-623.
8. Bellas, J., Granmo, K. and Beiras, R. (2005) Embryotoxicity of the antifouling biocide zinc pyrithione to sea urchin (Paracentrotus lividus) and mussel (Mytilus edulis). *Mar. Pollut. Bull.*, **50**, 1382-1385.
9. Goka, K. (1999) Embryotoxicity of zinc pyrithione, an antidandruff chemical, in fish. *Environ. Res.*, **81**, 81-83.
10. Kobayashi, N. and Okamura, H. (2002) Effects of new antifouling compounds on the development of sea urchin. *Mar. Pollut. Bull.*, **44**, 748-751.
11. Koutsaftis, A. and Aoyama, I. (2006) The interactive effects of binary mixtures of three antifouling biocides and three heavy metals against the marine algae Chaetoceros gracilis. *Environ. Toxicol.*, **21**, 432-439.
12. Mochida, K., Ito, K., Harino, H., Kakuno, A. and Fujii, K. (2006) Acute toxicity of pyrithione antifouling biocides and joint toxicity with copper to red sea bream (Pagrus major) and toy shrimp (Heptacarpus futilirostris). *Environ. Toxicol. Chem.*, **25**, 3058-3064.
13. Mochida, K., Ito, K., Harino, H., Onduka, T., Kakuno, A. and Fujii, K. (2008) Early life-stage toxicity test for copper pyrithione and induction of skeletal anomaly in a teleost, the mummichog (Fundulus heteroclitus). *Environ. Toxicol. Chem.*, **27**, 367-374.
14. Mochida, K., Amano, H., Onduka, T., Kakuno, A. and Fujii, K. (2011) Toxicity and metabolism of copper pyrithione and its degradation product, 2,2′-dipyridyldisulfide in a marine polychaete. *Chemosphere*, **82**, 390-397.
15. Mochida, K., Ito, K., Harino, H., Tanaka, H., Onduka, T., Kakuno, A. and Fujii, K. (2009) Inhibition of acetylcholinesterase by metabolites of copper pyrithione (CuPT) and its possible involvement in vertebral deformity of a CuPT-exposed marine teleostean fish. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, **149**, 624-630.
16. Okamura, H., Watanabe, T., Aoyama, I. and Hasobe, M. (2002) Toxicity evaluation of new antifouling compounds using suspension-cultured fish cells. *Chemosphere*, **46**, 945-951.
17. Onduka, T., Mochida, K., Harino, H., Ito, K., Kakuno, A. and Fujii, K. (2010) Toxicity of metal pyrithione photodegradation products to marine organisms with indirect evidence for their presence in seawater. *Arch. Environ. Contam. Toxicol.*, **58**, 991-997.
18. Howes, D. and Black, J.G. (1975) Comparative percutaneous absorption of pyrithiones. *Toxicology*, **5**, 209-220.
19. Gibson, W.B., Jeffcoat, A.R., Turan, T.S., Wendt, R.H., Hughes, P.F. and Twine, M.E. (1982) Zinc pyridinethione: Serum metabolites of zinc pyridinethione in rabbits, rats, monkeys, and dogs after oral dosing. *Toxicol. Appl. Pharmacol.*, **62**, 237-250.
20. Jeffcoat, A.R., Gibson, W.B., Rodriguez, P.A., Turan, T.S., Hughes, P.F. and Twine, M.E. (1980) Zinc pyridinethione: urinary metabolites of zinc pyridinethione in rabbits, rats, monkeys, and dogs after oral dosing. *Toxicol. Appl. Pharmacol.*, **56**, 141-154.