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Toxicology and Applied Pharmacology 207 (2005) S617 – S622

Toxicology and Applied Pharmacology

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Review

A comparison between integrated risk assessment and classical health/environmental assessment: Emerging beneficial properties

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Received 15 July 2004; revised 21 January 2005; accepted 21 January 2005
Available online 5 July 2005

Abstract

Both humans and wildlife are exposed to various types of halogenated organic compounds such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), typically old chemicals, and tris(4-chlorophenyl) methane (TCPM) and brominated flame retardants, some new chemicals, simultaneously. Classical risk assessment has evaluated health and ecological risks independently by experts from different disciplines. Taking into considerations the recent concerns about endocrine disrupting chemicals and the progress of research in related areas, we integrated and assessed data on exposure and potential effects in humans and wildlife. Comparisons were made for organ concentrations, body burdens of several organochlorine compounds (OCs), metabolic capacities between humans and various wildlife. When we integrate the knowledge on effects and exposure in humans and in wildlife, new insights were suggested about similarities and/or differences in potential effects among various human populations living on different foods and having different body burdens. Combining existing information with emerging knowledge of mechanisms of actions on endocrine disrupting chemicals after exposure to above chemicals during early developmental stages will further elucidate potential risks from exposure to those chemicals.

Keywords: DDT; *p*, *p*′-DDE; Tris(4-chlorophenyl) methane; PCB; Body burden

Contents

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0041-008X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2005.01.047

Introduction

For practical reasons, human health and environmental risk assessment methodologies have developed independently. However, with increased recognition of the need to protect both humans and the environment more effectively, an integrated approach to risk assessment that addresses real-life situations of multi-chemical, multimedia, multi-route and multi-species exposures is needed. Such an integrated approach would (1) improve the quality and efficiency of assessments through the exchange of information between human health and environmental risk assessors; and (2) provide more coherent inputs to the decision-making process. In response to this need, the UNEP/ILO/WHO, International Programme on Chemical Safety (IPCS), in collaboration with the U.S. Environmental Protection Agency, European Commission, and other international and national organizations, developed a working partnership to foster the integration of assessment approaches to evaluate human health and ecological risks. General framework of the integrated risk assessment was published previously (Suter et al., 2003). To substantiate this approach, an actual demonstration of the integrated risk assessment process is required to facilitate understanding and acceptance of the IPCS framework for integrated risk assessment by risk assessors and risk managers. This study composes a part of the above project to show potential benefits of the integrated risk assessment referring to existing health and ecological assessment documents on DDT and related compounds and to compare them with new approach applying some new data available.

Integration in problem formulation

The first step in the risk assessment process is the problem formulation, which identifies problems, needs of assessment, assessment objectives and the scope of assessment activities as well as the resources available for the assessment. Here, we describe needs for integrated approach with some organochlorine compounds (OCs) on the basis of new concern for endocrine disrupting compounds and recent progress of research in related areas.

Previous evaluations

Both humans and wildlife are exposed to various types of halogenated organic compounds such as PCBs, DDT and its metabolites like 1,1′-(2,2-dichloroethenyldiene)-bis(4-chlorobenzene) or *p*, *p*′-DDE, typically old chemicals, and TCPM and brominated flame retardants, some new chemicals, simultaneously. Classical risk assessment has evaluated health and ecological risks independently, typically assessed and reported by people from different disciplines. There are several international assessment or review documents for DDT and related compounds; however, there are no such documents found for TCPM. TCPM may originate from a variety of sources including production of synthetic high polymers, light-fast-dyes for acrylic fibres, anthelminthic drugs and formulations of agrochemicals, such as dicofol and technical DDT (Jarman et al., 1992). The International Programme on Chemical Safety (IPCS), a leading international body to evaluate chemical safety in terms of health and the environment, has evaluated DDT and its derivatives in two occasions independently, once for health aspects, and in another time for environmental aspects in its Environmental Health Criteria series (IPCS, 1979, 1989). The Joint FAO/WHO Meeting on Pesticide Residues (JMPR), an international expert committee associated with IPCS and also with the Codex Alimentarius, has reviewed toxicological aspects of DDT and its derivatives, several times from 1963 to 2000 (WHO, 2001). In the above IPCS's document for ecological risk assessment on DDT and its derivatives, it was mentioned that there is a fundamental difference in approaches between toxicologists and ecologists concerning the appraisal of the potential threat posed by chemicals (IPCS, 1989). For example, toxicologists are said to be preoccupied with any adverse effects on individuals, whether or not they have ultimate effects on performance or survival; however, ecotoxicologists are concerned primarily with the maintenance of population levels of organisms in the environment.

Impetus for the assessment

Recent concern about endocrine disrupting chemicals and progress of research in related areas has given us impetus to integrate and assess data on exposure and potential effects in humans and wildlife which may share similar exposure pathway and show potential effects to both humans and wildlife through similar mode of actions. Ecotoxicologist is said to be interested in effects on the performance of individuals in their reproduction and survival insofar as these might ultimately affect the

population size. Incidentally, a recent evaluation of DDT and its derivatives by the JMPR 2000 group, took into consideration the hormone modulating effects as one of the relevant effects for its toxicological evaluations.

Some evidence of endocrine disrupting potency of p, p′-DDE and TCPM

TCPM has similar chemical structures with *p, p′*-DDE (Fig. 1) which is known to cause eggshell thinning in pelicans, cormorants and other avian species (Cooke et al., 1976). TCPM showed binding activity to androgen receptors comparable to that of *p, p′*-DDE and the calculated K_i (0.62 μM) for TCPM was lower than reported K_is for antiandrogenic pesticide *p, p′*-DDE and vinclozolin in in vitro studies, although TCPM did not show effects on serum testosterone levels and morphology of testis in an in vivo study. An in vivo study showed that dietary dose of 12.4 mg/kg/day of TCPM to Sprague–Dawley rats elevated follicle stimulating hormone (FSH) in terminal blood samples (Foster et al., 1999).

Potential integration benefits

It might be useful to integrate and exchange information between ecotoxicologists and health toxicologists on the basis of new concern to the endocrine disrupting compounds and recent progress in the research in related areas to better answer needs of the risk managers and the societies. Formerly, the major effects of DDT and related compounds to humans were considered to be on the nervous system which is associated with the effect on the membrane in the nervous system (IPCS, 1979), while their effects to birds such as eggshell thinning (IPCS, 1989), were assessed independently with no consideration of possible links in effects between humans and wildlife. However, recent observations of endocrine disruptive effects of DDE and TCPM suggest that there might be potential risk not only to wildlife, but also to humans originating from common mechanism of actions and common route of exposure to these chemicals. Through integrating information of the health and ecotoxicological research, we may be able to effectively address possible difference and similarities between wildlife and humans.

Integration in the analysis plan

In this step of the risk assessment, one plans how to examine quantitatively the levels of exposure in addition to route of exposure and media for certain chemicals, while on the other hand, data of dose–response relationship for identified effects must be critically examined. New insights on similarities and differences in health and ecotoxicological effects in humans and wildlife will be obtained through integration of information in humans/experimental mammals and wildlife.

Exposure assessment

Identification of the source, environmental fate, exposure levels from various routes and ADME are major components in the exposure assessment step. There are fairly accurate estimates of the daily intake of DDT in several developed countries. Exposure aspects, such as environmental occurrence and fate, and route of exposure, are considered to be fairly common to both humans and wildlife, because food constitutes major route of exposure to DDT-type compounds for organisms of higher trophic levels although intake and kinetics may differ widely between them. In the case of persistent organohalogen compounds which are considered to cause hazardous effects chronically, body burden and tissue distribution may compose important parts in the exposure assessment. Therefore, we focused our approach of the integrated assessment to compare body burdens and tissue distributions in this study.

Tissue distribution and composition of OCs accumulation in humans

Amounts of OCs in adipose depot and in bile of humans were calculated from concentrations in adipose tissue multiplied by the weight of adipose tissue obtained from cadavers of patients in a hospital in Tokyo with

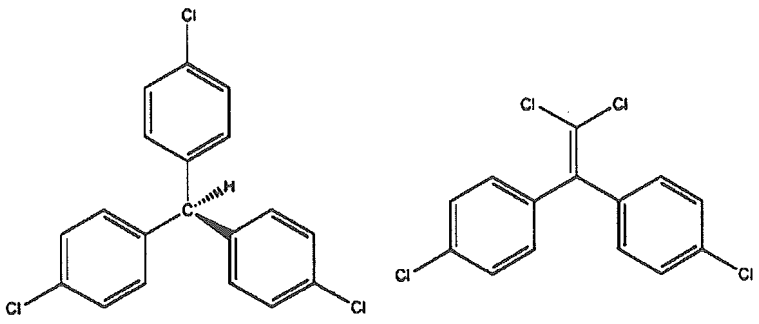

Fig. 1. Chemical structure of 1,1′-(2,2-dichloroethenyldiene)-bis(4-chlorobenzene) (*p,p′*-DDE) (right), and tris(4-chlorophenyl) methane (TCPM) (left).

informed consents from their families (Table 1) (Minh et al., 2001). Weight of adipose tissue (7700 g) was estimated from average body weight of patients (54 kg) multiplied by average fat content of the Japanese people (14.3%). Amount of OCs in bile (ng) was estimated assuming that maximally 1200 g bile is excreted per day, and lipid content of bile is 0.8% to obtain 9.6 g lipid in bile, which is then multiplied by OCs' concentrations in bile. Bile excretion rate is calculated as the ratio of the OC content in the bile to the amount of OCs in the adipose tissue. Since human adipose/bile and liver/bile concentration ratios are fairly close to each other, it suggests equilibrium between these tissues and biliary excretion of OCs (Table 2) (Minh et al., 2001). Low biliary excretion rates especially with *p, p′*-DDE, and TCPM indicated persistent accumulation of these compounds in the human bodies.

Comparison of OC patterns between humans and marine mammals

When compositions of OCs in Japanese human adipose tissues were compared to those in the blubber of cetaceans collected from Japanese coastal waters, humans have higher *p, p′*-DDE in total DDTs, and higher oxychlordane in total chlordanes than marine mammals (Minh et al., 2000; Tanabe, 2002). This suggests that there exists some difference between humans and certain wildlife in that some marine mammals which are devoid of phenobarbital-induced mixed function oxidases (MFOs), and having lower content of methylcholanthrene-induced MFOs, have lower capacities to metabolize OCs.

Potential integration benefits

Organ concentrations, body burdens of several environmentally polluting compounds, and metabolic capacities were compared between humans and various wildlife. Above example illustrates that simple estimation from combination of fat content of the body, octanol–water partition coefficient of the compound, and environmental concentrations is not enough, but integration of other information such as on difference of metabolic capacities among various species is important in explaining large species differences in body burden among species. New insights were suggested about potential differences and similarities in effects not only between humans and wildlife, but also among various human populations living on different foods (see below) and having different body burdens from knowledge thus far integrated regarding OC compounds. This information together with emerging knowledge on mechanisms of actions of endocrine disrupting chemicals after exposure during early developmental stage will further elucidate potential risks to both humans and wildlife from exposure to those chemicals.

Table 1
Concentrations of several organochlorine compounds in human adipose tissue, liver and bile from Japan (ng/g lipid wt)[a]

Sample no.	Fat (%)	TCPM	PCBs	*p, p′*-DDE	DDTs
Adipose tissue					
Mean	63	18	1904	2321	2300
Range	46–76	2.7–44	230–6600	150–7900	160–8100
Geometric mean	65	16	1514	1413	1514
Liver					
Mean	7.3	7	1237	1299	1600
Range	2.5–14	1.1–20	240–2900	120–5600	140–5800
Geometric mean	6.3	5.01	1023	692	891
Bile					
Mean	0.8	17	2871	642	880
Range	0.5–1.5	<5–62	1800–4600	130–1800	160–1900
Geometric mean	0.74	14.5	2754	631	676

[a] Samples were taken from 3 female and 4 male persons.

Table 2
Mean concentrations ratios of organochlorines between adipose tissue, liver and bile in humans from Japan (n = 7)

	TCPM	PCBs	*p, p′*-DDE
Concentration ratio			
Wet weight basis			
Adipose/liver	24	14	14.0
Adipose/bile	130	57	212
Liver/bile	5.5	4.1	15.2
Lipid weight basis			
Adipose/liver	3.19	1.48	2.04
Adipose/bile	1.1	0.54	2.20
Liver/bile	0.35	0.37	1.10

Lipid content in adipose tissue: 635%, liver: 7.3%, bile: 0.8%.

Dose–response assessment

The JMPR group in 1984 evaluation (FAO, 1985) estimated a no-observed-adverse-effect-level (NOAEL) for nongenotoxic carcinogenicity from a rat 2-year study as 6.2 mg/kg bw. per day, and also they concluded then, that there was no firm evidence that DDT has any reproductive or teratogenic effects. They also estimated 0.25 mg/kg bw. per day as an overall NOAEL for humans for no changes in liver functions as observed in workers exposed to DDT of 0.05–0.25 mg/kg bw. per day. However, the 2000 JMPR evaluations (WHO, 2001) noted based on new findings, that activation of estrogen receptors and inhibition of androgen receptors may be mechanisms of the action of DDT-related compounds which led to the observed perturbation of reproductive function. NOAEL of 1 mg/kg bw. per day for developmental effects was estimated based on observations of decreased ovarian weights, cystic ovaries, loss of corpora lutea, infertility, premature puberty, altered onset of vaginal opening, tail anomalies, and increased pup mortality rates. On the other hand, developmental effects, such as eggshell thinning are known to be caused by *p,p′*-DDE (Cooke et al., 1976), and some mammals such as bat which shows marked seasonal cycles in fat content are affected by DDT and its metabolites, although exact mechanisms were not known (IPCS, 1989). Recently, potential effects of

endocrine disrupting chemicals after exposure during critical stages of development to them at low dose levels, which were not known before, were suggested (IPCS, 2002). In addition, body burdens of TCPM, DDT, DDE and PCB on the lipid basis were shown to be sometimes similar between fish-eating human populations and seals or birds, in which seals were collected during outbreak of unusual mortality in Caspian sea caused probably by canine distember virus to immune function deteriorated animals (Minh et al., 2001, Kajiwara et al., 2002, Kunisue et al., 2002).

Potential integration benefits

Old findings of eggshell thinning suggested that this effect might be induced by hormonal disturbance. By the accumulation of knowledge in in vitro and in vivo studies in experimental animals together with supporting evidence from wildlife observations, a new evaluation was deduced. Recent reports (for example, Miyazaki et al., 2004) suggest that some organochlorine compounds and/or their metabolites can exert neurobehavioral or immune effects at very low dose levels. Investigations of this kind of effects which can be induced via disturbance of regulations of the biological process at molecular and cellular levels may change current estimation of risks by these compounds. Here again, we can benefit from the integration in the risk assessment process.

Integration in risk characterization

Formerly, an acceptable daily intake (ADI) of 0–0.02 mg/k bw. was allocated in 1984 for combination of DDT, DDD, and DDE, principally based on human studies where no overall change was observed in liver functions in workers exposed to 0.05–0.25 mg/kg bw., and it was converted to provisional tolerable daily intake in 1994 because of the lack of reliable data on the consequence of exposure to these compounds. However, the JMPR 2000 evaluation derived a Provisional Tolerable Daily Intake (PTDI) of 0.01 mg/kg bw. through its toxicological evaluations on the basis of the NOAEL of 1 mg/kg/day for the developmental toxicity in rats and a safety factor of 100 as described above (WHO, 2001).

Comparison of organochlorine residues in human liver from several countries

Distribution of several OCs in human liver and their ratios were compared (Table 3). PCB concentration is highest in Greenlanders (42,000 ng/g lipid), followed by Norwegians (1900 ng/g lipid), Japanese, Finnish and Swedish with the lowest average concentrations in Americans and Canadians. Very high concentrations in Greenlanders are probably reflecting their food intake pattern where they eat much meat of marine fish or mammals which accumulate high OCs in their adipose tissues. However, compared to PCB concentrations, DDT concentration was highest among Americans and Canadians (3600 ng/g lipid), followed by Greenlanders (2900 ng/g lipid), Japanese, Swedish, Norwegians, and Finish with the lowest average concentrations in Italians (310 ng/g lipid). The PCB/DDT ratios were the highest in Greenlanders (14.4), medium in Finish (2.2), low in Japanese (0.75), and very low in Americans and Canadians (0.08). Since some PCBs (coplanar PCB) are known to exert dioxin-like effects via arylhydrocarbon receptors, or developmental effects possibly via perturbation of thyroid and retinoid metabolism, while *p,p′*-DDE, a metabolite of DDT was shown to exert its effects possibly as an antiandrogen. This wide difference in the amount and the ratio of OCs accumulations among various populations, not only reflects their food intake patterns, but also suggests that OCs may possibly exert different health effects among them. To understand risks better from the exposure to these OCs and for efficient risk management thereof, we need to integrate our knowledge on OC exposure and their possible effects further.

Table 3
Comparison of organochlorine residues (ng/g lipid wt) in human liver from different countries

Country	Year	PCBs	DDT	PCBs/DTT	References
Japan	1999	1023	891	1.15	Present study
Finland	1982–83	1100	550	2.2	Minh et al. (2001)
Norway	1977	1900	800[a]	2.4	ibid.
Sweden	1997	1100	840[a]	1.31	ibid.
Italy	1989	nd	310[b]	–	ibid.
Greenland	1992–94	42,000	2900[c]	14.4	ibid.
US and Canada	1980s	280	3600	0.08	ibid.

Abbreviations: nd, not determined.
[a] *p, p′*-DDE only.
[b] Sum of *p, p′*-DDE, *p, p′*-DDT and *o, p′*-DDT.
[c] Sum of *p, p′*-DDT and *p, p′*-DDE.

Potential integration benefits

We may be able to obtain a better understanding of our exposures and potential effects to both human health and wildlife by integrating our knowledge and information available to us. We need to compare and understand what kind of risk from what types of chemicals via which route of exposure may potentially threat our health and wildlife, and efficiently allocate our resources to better cope with the problems. Integrated risk assessment as shown in this study, will help us judge based on holistic view combined with mechanistic knowledge in the background. Sekizawa et al. (2003) showed another good example of integrated risk assessment using the case of organotin compounds, in which they pointed out that understanding of the basic mechanism behind the apparently independent phenomena in wild life (imposex which is penis development in female gastropods) and experimental mammals (immune toxicity), will elucidate a link between them. Recent report of a

finding (Kaneko et al., 2004) that organotin compounds can be bound with high affinity to retinoid receptors which play important roles in transcriptional regulation of diverse cellular functions, will substantiate this suggestion. A case study for the persistent organic pollutants was published also previously (Ross and Birnbaum, 2003).

Conclusions

Integrated knowledge on chemical properties and on similarities and/or differences in toxicokinetics of endocrine disruptors will give us new insights on potential effects among various populations living on different foods and having different body burdens. Endocrine disruptors are a matter of concern and have impacts on both human health and wildlife. Parallel studies of toxicokinetics and dynamics on humans and wild life, and integrated risk assessment based on them will tell us how and to what extent the environmental pollution is posing risk to human health and wildlife. Integrated risk assessment thus will give us better understanding and consistent basis on effective environmental risk management from both health and environmental protection points of view.

Acknowledgments

This study is a part of the authors' cooperative studies on the Potential risk to future generations from exposures to complex chemical mixtures, supported by the Ministry of the Environment, Japan (2000–2002).

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Effects of Nonylphenol and Triclosan on Production of Plasma Vitellogenin and Testosterone in Male South African Clawed Frogs (*Xenopus laevis*)

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We investigated the effects of nonylphenol (NP) and triclosan (TCS) on production of vitellogenin (Vg), testosterone (T), and hepatic cytochrome P450 1A and 2B activities in male South African clawed frogs (*Xenopus laevis*). In a 14-d waterborne exposure test, no significant differences in the level of plasma Vg synthesis in male frogs were observed among the control, 10, 50, and 100 μg/l NP and 20, 100, and 200 μg/l TCS treatment groups. Intraperitoneal injection of male frogs with 2, 20, and 200 μg/g body weight NP resulted in no significant differences in plasma Vg levels among the control and all treatment groups. However, the levels of plasma Vg in all TCS treatment groups (intraperitoneal injection of 4, 40, and 400 μg/g body weight) were lower than that in the solvent control group, and male frogs injected with high doses of NP or TCS had lower T levels than the control group. No significant differences in hepatic cytochrome P450 1A and 2B activities were observed among the all treatment groups. Male frogs injected with 20 μg/g body weight of estradiol-17β had significantly higher plasma Vg levels than the control group. These results suggest that profiles of plasma Vg and T production in male *Xenopus laevis* could be useful biomarkers for detecting hormonally active agents.

Key words *Xenopus laevis*; vitellogenin; nonylphenol; triclosan

Recently, a number of studies have been performed worldwide to examine endocrine-disrupting chemicals (EDCs) and their interactions with the development and function of various systems in animals and humans.[1—3] Among these EDCs, alkylphenol polyethoxylate non-ionic surfactants are used in the manufacture of cleaning agents, cosmetics and food products, as well as in plastic polymerization processes. Nonylphenol ethoxylates have predominantly been used, amounting to about 80% of the production of alkylphenol surfactants. In a recent study, nonylphenol (NP) had significant effects on the reproductive potential of medaka (*Oryzias latipes*) at concentrations as low as 17.7 μg/l,[4] and 50% of the male fish in the 50 μg/l treatment and 86% of the males in the 100 μg/l treatment developed testis-ova, an intersex condition characterized by both testicular and ovarian tissue in the gonad.[5] On the other hand, triclosan (TCS, 2,4,4′-trichloro-2′-hydroxydiphenyl ether) is widely used as an antibacterial agent in liquid toothpaste, soap, shampoo, and cosmetics, and is frequency found in wastewater effluent. Water samples collected near the outfall of a wastewater treatment plant in Rhode Island, U.S.A., showed 10—20 μg/l of TCS in the effluent and 80—100 μg/g of TCS in the sediment.[6,7] Our previous study suggests that TCS has high toxicity on the early life stages of medaka, and that the metabolite of TCS may be a weak estrogenic compound in male medaka but with no adverse effect on reproductive success (such as fecundity and fertility) and offspring.[8] Moreover, Hanioka *et al.*[9,10] reported that 7-benzyloxyresorufin *O*-debenzylase (BROD) and 7-pentoxyresorufin *O*-dependentylase (PROD) activities, which are associated with CYP2B1 activity, were remarkably induced by all doses of TCS in rats. Their results suggested that TCS induces the P450 isoforms of the CYP2B subfamily in the rat liver, and that the induced P450 isozymes were closely related to the toxicity of TCS or its chlorinated derivatives. However, there is no information about the effects of these chemicals on the hormonally responses of amphibian.[11]

The South African clawed frog (*Xenopus laevis*) is sensitive to environmental chemicals as it spends all the life stages (egg, larva, and adulthood) in water. Hayes *et al.*[12] examined the effects of atrazine on sexual development in *X. laevis*, and reported that atrazine (> or =0.1 μg/l) induced hermaphroditism and demasculinized the larynx of exposed males (> or =1.0 μg/l), and male *X. laevis* suffered a 10-fold decrease in testosterone levels when exposed to 25 μg/l atrazine. Therefore, in the present study, we used *X. laevis* as a test organism, and investigated the effects of NP and TCS on production of plasma vitellogenin (Vg, egg yolk protein precursor), steroid hormone testosterone (T) synthesis, and hepatic CYP1A and CYP2B, as measured by the ethoxyresorufin *O*-deethylase (EROD) or PROD activities in male *X. laevis*. To our knowledge, this is first study on the effect of TCS in male *X. laevis*.

MATERIALS AND METHODS

Test Chemicals TCS (>98.0% purity) was obtained from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. NP (technical grade; mixture of ring and chain isomers) was obtained from Aldrich Chemical Company Inc., Tokyo, Japan. These chemicals were dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries) and propylene glycol (Wako Pure Chemical Industries) to prepare test solutions.

Test Organism Male *X. laevis* (body weight; approximately 50—60 g) were purchased from Kato S Science,

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Chiba, Japan, and have been maintained in glass tanks in our laboratory under 16 h light–8 h dark photoperiod at 24±1 °C. The frogs were fed a commercial diet (Kato S Science) once daily during the acclimatization period for 7 d.

Exposure Design Frogs were exposed to the chemicals either *via* water or intraperitoneal injection. In the waterborne exposure, each group of frogs (six frogs per treatment) was exposed to the nominal concentrations of 10, 50, and 100 μg/l NP, and 20, 100, and 200 μg/l TCS dissolved in dechlorinated tap water for 14 d. The control frogs were exposed to the solvent carrier only (DMSO 0.1 ml/l), and the positive control frogs were exposed to the nominal concentration of 1 μg/l of estradiol-17β (E2). The exposure periods of these chemicals were selected on the basis of the results of a previous study.[13] During the experimental period, the exposure water in the tanks was changed every 24 h. Blood samples were then taken at the end of the exposure period.

In the intraperitoneal exposure, each group of frogs (six frogs per treatment) was injected with 2, 20, and 200 μg/g body weight NP, and 4, 40, and 400 μg/g body weight TCS dissolved in propylene glycol. The control frogs were injected with the solvent carrier only (propylene glycol), and the positive control frogs were injected with 20 μg/g body weight of E2. After the injection, frogs were transferred to glass tanks containing dechlorinated tap water, and maintained for 7 d. Blood samples were then taken at the end of the exposure period.

In both exposure systems, each group of frogs was kept in a 30-l glass tank maintained under 16 h light–8 h dark photoperiod at 24±1 °C, and fed 0.3% body weight of a commercial diet every day during the experimental period.

Preparation of Blood and Microsome Samples Frogs were weighed and blood samples were taken from the caudal vasculature with heparinized syringe and needle. Blood samples were transferred into a centrifuge tube, and centrifuged at 6000×*g* for 10 min. The plasma was then stored at −80 °C until assayed. All preparative procedures were carried out at 4 °C.

The liver was homogenized with 4 volumes of 0.25 M sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. Preparation of the 105000×*g* soluble fraction and microsomes was carried out by the procedures previously described by Arizono *et al.*[14] and Ariyoshi *et al.*,[15] respectively. Microsomal protein content was determined according to Lowry *et al.*[16] using bovine serum albumin as a standard. The microsomal samples were stored at −80 °C until use. All preparative procedures were carried out at 4 °C.

Measurement of Plasma Vg Plasma Vg levels in male frog were measured using enzyme-linked immunosorbent assay (ELISA) in a Vg assay kit (Japan EnviroChemicals, Osaka, Japan) specifically for frog developed by Mitsui *et al.*[13] The measurement of plasma Vg was performed according to the manufacture's procedure. Purified frog Vg (1—1000 ng/ml) was used as a standard, and Vg in diluted samples was measured in duplicate. The assays were performed at room temperature. Concentrations of Vg in plasma samples were calculated from the linear part of the log-transformed frog Vg standard curve. The detection limit of Vg in the present study was 1 ng/ml.

Measurement of Plasma T Plasma T levels were measured by an ELISA as described by Ishibashi *et al.*[17] T was extracted from blood plasma three times using ten volumes of diethyl ether. The ether was evaporated under nitrogen, and the sample was reconstituted with assay buffer. Each sample was analyzed in duplicate for T corrected for extraction efficiencies of 90%. The minimum concentration detectable was 50 pg/ml for T. In this ELISA system, inter-assay and intra-assay coefficients of variation were less than 10% for plasma T.

Measurement of Hepatic EROD and PROD Activities EROD and PROD activities in frog liver microsomes were measured by dealkylation of ethoxyresorufin and pentoxyresorufin, respectively, and detection of the resulting resorufin by high-performance liquid chromatography (HPLC) with fluorescent detection as described by Tatarazako *et al.*[18]

Statistical Analysis All statistical analyses were performed using Stat View J 5.0 (SAS Institute Inc., Cary, North Carolina, U.S.A.). All experimental data were checked for assumptions of homogeneity of variance across treatments using a Bartlett test. When the assumptions were met, data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison tests.[19] When no homogeneity was observed in the data, nonparametric Kruskal–Wallis test was used, followed by a Mann–Whitney *U* test with Bonferroni's adjustment.[20] Differences were considered significant at $p<0.05$ or $p<0.01$.

RESULTS AND DISCUSSION

In this study, we investigated the effects of NP and TCS on production of Vg, T, and hepatic EROD or PROD activities in male *X. laevis*. In the waterborne exposure, there were no significant differences in body weight among the solvent control, NP- or TCS-treated groups (ranging from 51.7 to 62.8 g). In addition, the intraperitoneal injection, there were no significant differences in body weight among the solvent control and NP- or TCS-treated groups (ranging from 53.3 to 62.0 g); however, all the animals injected 200 μg/g body weight of NP died during the experimental period.

In the waterborne exposure, there were no significant differences in plasma Vg levels among the NP- or TCS-treated groups (Fig. 1). The 10 μg/l NP tested in this study was often detected in the aquatic environment, and 50 and/or 100 μg/l NP caused the induction of Vg in fish.[21—23] On the other hand, water samples collected near the outfall of a wastewater treatment plant in Rhode Island, U.S.A., showed 10—20 μg/l of TCS in the effluent.[6] These results indicate that NP or TCS did not have any estrogenic effects on adult male frog at the concentrations detected in the aquatic environment. Furthermore, high levels of E2-1 μg/l did not induce plasma Vg in male frog in the present study (Fig. 1), although it is known that endogenous estrogen such as E2 induced the plasma and/or hepatic Vg in fish.[24,25]

In the intraperitoneal injection, we could not demonstrate the induction of plasma Vg in male *X. laevis* at NP concentrations tested in this study as well as in result of water exposure (Fig. 2). Van Wyk *et al.*[11] reported that no induction of plasma Vg in male *X. laevis* treated with 100 μg NP/g/week. Therefore, NP may not have estrogenic effect in adult male *X. laevis*. Moreover, male frogs injected with high doses of NP had lower T levels than the control group, although no statistically significant difference was determined because a

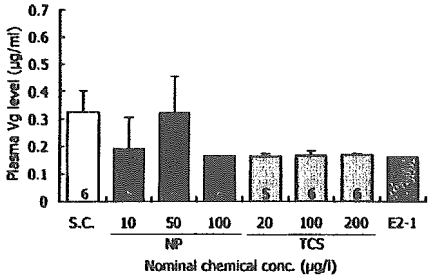

Fig. 1. Levels of Plasma Vitellogenin (Vg) Concentration of Male Frogs (*Xenopus laevis*) Treated with Nonylphenol (NP) and Triclosan (TCS) in Water Exposure for 14 d

The control frogs (S.C.) were exposed to the solvent carrier only (DMSO 0.1 ml/l), and the positive control frogs were exposed to the nominal concentration of 1 μg/l of estradiol-17β (E2). Data represent at the mean±S.D. The number in each bar represents the number of individuals.

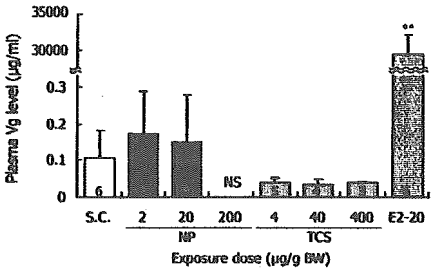

Fig. 2. Levels of Plasma Vitellogenin (Vg) Concentration of Male Frogs (*Xenopus laevis*) Treated with Nonylphenol (NP) and Triclosan (TCS) in Intraperitoneal Injection

The control frogs (S.C.) were injected with the solvent carrier only (propylene glycol), and the positive control frogs were injected with dose of 20 μg/g body weight of estradiol-17β (E2). NS; no samples. ** Significant difference compared to the control frogs ($p<0.01$). Data represent at the mean±S.D. The number in each bar represents the number of individuals.

large individual variation was observed (Fig. 3). Similar observations have been reported in *X. laevis* exposed to NP.[11] It might be that NP inhibited an aspect(s) of steroid release and/or synthesis common to T in male *X. leaevis*. However, Okoumassoun *et al.*[26] assessed the estrogenic activity of the organochlorine pesticides *o,p'*-DDT, dieldrin, aldrin, heptachlor, mirex and DDT in rainbow trout hepatocyte cultures using Vg. Heptachlor and mirex did not induce Vg. They suggested that the EC_{50} value for inhibition of estrogen receptor binding by heptachlor was cytotoxic for hepatocytes in culture, and this could in part explain the lack of Vg response observed with this compound at the concentrations tested. In this study, although there were no significant differences in body weight among all treatment groups, all frogs injected with 200 μg/g body weight of NP died during the experimental period. Thus, no Vg production in male frogs exposed to NP may cause lethal toxicity.

The levels of plasma Vg in all TCS treatment groups were lower than that in the solvent control group in the intraperitoneal injection, but no statistically significant difference was determined (Fig. 2). In addition, male frogs injected with high doses of TCS had lower T levels than the control group, but no statistically significant difference was determined (Fig. 3). Panter *et al.*[27] reported that while the pharmaceutical antiestrogen ZM 189,154 had no impact on somatic endpoints in juvenile fathead minnow (*Pimephales promelas*), this substance caused a reduction in Vg concentrations,

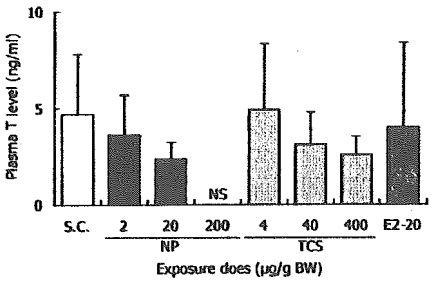

Fig. 3. Levels of Plasma Testosterone (T) Concentration of Male Frogs (*Xenopus laevis*) Treated with Nonylphenol (NP) and Triclosan (TCS) in Intraperitoneal Injection

The control frogs (S.C.) were injected with the solvent carrier only (propylene glycol), and the positive control frogs were injected with dose of 20 μg/g body weight of estradiol-17β (E2). NS; no samples. Data represent at the mean±S.D. The number of each treatment group is shown in Fig. 2.

which is consistent with its mode of action as an estrogen receptor antagonist. Recent studies have demonstrated changes in fin length and non-significant trends in sex ratio of medaka (*Oryzias latipes*) exposed to 1—100 μg/l TCS detected in aquatic environment, and have suggested that TCS has weak androgenic and antiestrogenic potency.[28] Our previous study also suggests that the metabolite of TCS may be a weak estrogenic compound in male medaka.[8] Therefore, the reduction of plasma Vg in this study may be due to the antiestrogenic effects of TCS in male *X. laevis*.

In the present study, male frogs injected with 20 μg/g body weight of E2 had significantly higher plasma Vg levels than the solvent control group (Fig. 2). Van Wyk *et al.*[11] also reported that treatment with 10 μg/g/week of E2 induced plasma Vg synthesis in male *X. laevis*. These results suggest that induction of plasma Vg in male *X. laevis* could be a suitable biomarker for the evaluation of estrogenic activity of EDCs.

Fujita *et al.*[29] reported molecular cloning and sequence analysis of cDNAs coding for 3-methylcholanthrene-inducible CYP1A isoform in *X. laevis* liver. In contrast, induction of hepatic CYP2B by phenobarbital (PB) has been reported in semi-aquatic frog (*Rana pipiens*).[30] Therefore, the determinations of EROD and PROD activities in frog are important markers of toxicological research.[30] Hanioka *et al.*[9,10] reported that BROD and PROD activities in rats, were induced by all doses of TCS, suggesting that TCS induced the hepatic P450 isoforms of the CYP2B subfamily. We also assessed the induction of EROD and PROD activities in hepatic microsomes from male frog exposed to TCS but found no difference between the EROD and PROD activities among the TCS treatment groups (Fig. 4). Although PB induction of CYP2B has not been clarified in *X. laevis*, it is unclear whether our results are due to physiological differences in the induction of hepatic CYP2B between *X. laevis* and *R. pipiens*. Further experiments in the laboratory are required to evaluate CYP2B induction in *X. laevis* exposed to various CYPs inducers.

Acknowledgments We should like to express our grateful thanks to Mr. Noriaki Santo and Dr. Osamu Tooi (Towa Kagaku Co., Ltd., Hiroshima, Japan) for technical advice. The authors would like to thank Dr. Charles Sagoe (Towa Kagaku Co., Ltd., Hiroshima, Japan) for critical reading of

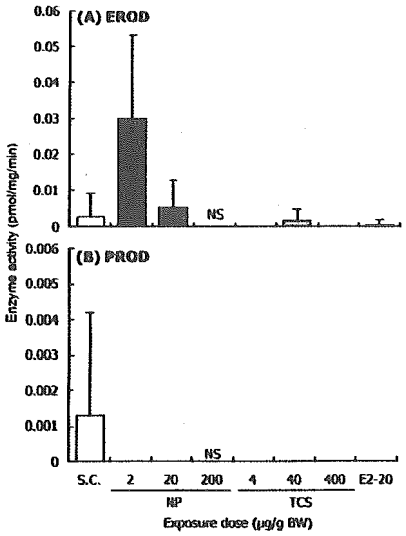

Fig. 4. EROD and PROD Activities in Microsome of Male Frogs (*Xenopus laevis*) Treated with Nonylphenol (NP) and Triclosan (TCS) in Intraperitoneal Injection

The control frogs (S.C.) were injected with the solvent carrier only (propylene glycol), and the positive control frogs were injected with dose of 20 μg/g body weight of estradiol-17β (E2). NS; no samples. Data represent at the mean±S.D. The number of each treatment group is shown in Fig. 2.

manuscript. We thank Towa Kagaku Co., Ltd. (Hiroshima, Japan) and Japan EnviroChemicals (Osaka, Japan) for providing the *X. laevis* Vg ELISA kit. This study was supported in part by funds from the Environment Agency, Japan.

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Study of 1,4-Dioxane Intake in the Total Diet

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(Received April 25, 2005; Accepted May 9, 2005)

1,4-Dioxane is a newly added compound to the water quality standards in Japan that were revised in 2003. In order to estimate the contribution of 1,4-dioxane in drinking water to the total exposure in humans, it is necessary to take into account the quantity of the compound in food. In an earlier study, we measured the intake of 1,4-dioxane in food based on the average consumption of food in the Kanto area.[1] The total daily intake of 1,4-dioxane was calculated to be 0.440 μg. In the present study, we investigated the intake of 1,4-dioxane from food by sampling meals from 3 days from 3 homes in 9 prefectures, respectively. 1,4-Dioxane was extracted from 20 g of homogenates of mixed meals using the steam distillation, concentrated by a solid phase cartridge and then measured using gas-chromatography/mass spectrometry. The detection limit of the analysis was 2 μg/kg. No 1,4-dioxane was detected in 26 samples, while 3 μg/kg was detected in one sample. In this sample case, the daily intake of the 1,4-dioxane was calculated as 4.5 μg that represented 0.56% of the total daily intake (TDI) (4.5 μg/{16 μg/kg body weight/day × 50 kg}).

Key words —— 1,4-dioxane, total diet, risk, total meal

INTRODUCTION

1,4-Dioxane has been classified as a carcinogenic compound by both the USA Environmental Protection Agency[2] and the International Agency for Research on Cancer (IARC).[3] Long-term oral administration of 1,4-dioxane has been shown to cause tumors in the liver and gallbladder in guinea pigs,[4] and in the nasal cavity and liver of rats.[5–8] Studies in mice using a two-stage carcinogenic test have demonstrated 1,4-dioxane also has promoter activity.[9]

In 2003, 1,4-dioxane was added to the revised water quality standards in Japan. The compound is used extensively as an industrial solvent and is also added as a stabilizer to chlorinated solvents.[10] 1,4-Dioxane escapes to the aquatic environment and after discharging into the atmosphere returns to the surface as rainwater. As a result of its low adsorption to soil, 1,4-dioxane then permeates into the groundwater causing long term water pollution. As a consequence, 1,4-dioxane has the potential to cause widespread contamination of the environment and it is therefore important when evaluating exposure to the compound that every potential route of contamination is taken into account. Although there are several reports of 1,4-dioxane being detected in the environment,[11–13] there have been few reports on the content of 1,4-dioxane in food. Levels of 1,4-dioxane between 0.2 and 1.5 mg/l were detected in tap water samples collected between 1995 and 1996 from six cities in Kanagawa prefecture, Japan,[14] this finding raises the possibility that food may also have become contaminated. As there have been few reports on the contents and intake of 1,4-dioxane in food, in order to safeguard human health in Japan it is important to determine the intake levels of 1,4-dioxane in food.

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In an earlier study we measured the intake of 1,4-dioxane in food based on the average intake of food in the Kanto area of Japan as reported by the Ministry of Health, Labor and Welfare.[1] The total daily intake of 1,4-dioxane was calculated to be 0.440 μg. An intake of this magnitude corresponded to 0.055% of the calculated total daily intake (TDI) of 16 μg/kg body weight/day. In this paper, we extended these investigations by measuring the intake of 1,4-dioxane from meals.

MATERIALS AND METHODS

Chemicals —— 1,4-Dioxane was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), 1,4-dioxane-d_8 from Sigma-Aldrich Co., Ltd. (Tokyo, Japan), dichloromethane from Kanto Chemical Industry Co., Ltd. (Tokyo, Japan), ethanol from Katayama Chemical Industry Co., Ltd. (Osaka, Japan), acetonitrile and acetone from Wako Pure Chemical Industry Co., Ltd. (Osaka, Japan), and the antifoaming agent silicon TAS730 from Toshiba Silicon Co., Ltd. (Tokyo, Japan). All solvents were of the highest reagent grade. Purified water was prepared using a Milli-Q water purification PSS20 system (Millipore Corp., Bedford, MA, U.S.A.).

Preparation of Standard Solutions —— Standard solutions of 1,4-dioxane and 1,4-dioxane-d_8 were prepared in dichloromethane from stock solutions of 1 mg/ml 1,4-dioxane and 10 mg/ml 1,4-dioxane-d_8 as described previously.[1]

Preparation of Food Samples —— Samples of meals from 3 days were collected daily from 3 homes in 9 prefectures. The samples of the three meals cooked each day were added to samples of between-meal snacks and drink, followed by mixing and homogenization. The homogenates were then stored in glass bottles with silicon seals and kept frozen at –20°C until analyzed.

Extraction of 1,4-Dioxane —— 1,4-Dioxane was extracted from 20 g of each mixed meal homogenate using steam distillation as described previously reported.[1] Briefly, the samples were added to 150 ml of purified water and 100 μl of 2 μg/ml 1,4-dioxane-d_8 solution and then extracted, followed by concentration using a solid phase cartridge.

GC/MS Analysis —— Gas-chromatography/mass spectrometry (GC/MS) analysis was carried out using an Agilent 6890/5973N instrument (Agilent Technologies Inc., Palo Alto, CA, U.S.A.) instrument with an SPB-624 capillary column (60 m × 0.25 mm i.d. × 1.4-μm film thickness) (Sigma-Aldrich Co., Ltd.), as reported previously.[1] In the selected ion monitoring (SIM) mode, the monitoring ions were 58 and 88 for 1,4-dioxane and 64 and 96 for 1,4-dioxane-d_8. A calibration curve was prepared from the ratio of the peak height of 1,4-dioxane and 1,4-dioxane-d_8.

RESULTS AND DISCUSSION

Detection Limit in Food Samples

The minimum detection level of 1,4-dioxane added as an internal standard was 0.04 μg/l (S/N = 10), while the minimum detection limit of 1,4-dioxane in the prepared mixed meal was 2 μg/kg calculated using the following formula: (0.04 μg/l × 1 ml)/ 20 g = 0.002 μg/g = 2 μg/kg. In this formula, 1 ml represents the final volume of the GC/MS analysis and 20 g represents the weight of the mixed meal homogenate.

Recovery Test of 1,4-Dioxane

The concentration of 1,4-dioxane in the purified water used in the analysis was less than 0.04 μg/ml. After the addition of 0.2 μg of 1,4-dioxane and 1 μg of 1,4-dioxane-d_8 to 4 g of the prepared food samples, the recovery rate of 1,4-dioxane was obtained using the method described in the MATERIALS AND METHODS. The recovery rate of 0.2 μg of 1,4-dioxane was between 99 and 111% in the 12 food groups.[1] These results indicate that extraction of the compound from any food was efficient and met the requirements for this study.

Content of 1,4-Dioxane in the Mixed Meal Samples

The extraction of 1,4-dioxane from each of the 20 g prepared mixed meal samples was carried out according to the method described in the MATERIALS AND METHODS. Table 1 shows the content of 1,4-dioxane in the 27 mixed meal samples. 1,4-Dioxane was not detected in 26 of these samples but was detected in the remaining sample of a meal which collected on the first day from home C in the Nagano prefecture. The content of 1,4-dioxane detected in this sample was 3 μg/kg. Based on data reported by the Ministry of Health, Labor and Welfare the total weight of meals consumed each day is approximately 1.5 kg. Therefore, in the case of the positive sample the daily intake of the 1,4-dioxane was calculated to be 4.5 μg.

Table 1. Content of 1,4-Dioxane in the Mixed Meal Samples

Sample Site	Home Name	Day	Content (μg/kg)	Sample Site	Home Name	Day	Content (μg/kg)
Hokkaido	A	1st day	ND	Nagano Prefecture	A	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	B	1st day	ND		B	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	C	1st day	ND		C	1st day	3
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
Miyagi Prefecture	A	1st day	ND	Hyogo Prefecture	A	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	B	1st day	ND		B	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	C	1st day	ND		C	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
Tokyo	A	1st day	ND	Kagawa Prefecture	A	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	B	1st day	ND		B	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	C	1st day	ND		C	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
Aichi Prefecture	A	1st day	ND				
		2nd day	ND				
		3rd day	ND				
	B	1st day	ND				
		2nd day	ND				
		3rd day	ND				
	C	1st day	ND				
		2nd day	ND				
		3rd day	ND				

There is evidence that long-term oral administration of 1,4-dioxane in rodents causes hepatic and nasal cavity tumors in rodents,[8–12] and accordingly the IARC has classified 1,4-dioxane as a group 2B carcinogen.[6] With regard to a cancer endpoints, a TDI of 16 μg of 1,4-dioxane/kg body weight/day has been calculated by applying an uncertainty factor of 1000 to the level of 16 μg/kg body weight/day at which no adverse effects were observed in a long-term study of drinking water in rats.[15,16] This uncertainty factor incorporates 100 for inter- and intraspecies variation and 10 for nongenotoxic carcinogenicity. An intake of 4.5 μg of 1,4-dioxane corresponded to 0.56% of the TDI (4.5 μg/{16 μg/kg body weight/day × 50 kg}). As this proportion was the highest value in this investigation, we consider all

other proportions would be equal to or less than 0.56%. We therefore conclude that the intake of 1,4-dioxane from food appears to be very low and that this value does not increase the risk of carcinogenicity.

Acknowledgements This work was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan.

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Short communication

Analysis of herbicides in water using temperature-responsive chromatography and an aqueous mobile phase

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Received 16 July 2004; received in revised form 26 January 2005; accepted 31 January 2005

Abstract

A simple and rapid method has been developed for herbicides in water using temperature-responsive liquid chromatography (LC) and a column packed with poly(*N*-isopropylacrylamide) (PNIPAAm), a polymer anchored on the stationary-phase surface of modified silica. PNIPAAm reversibly changes its hydrophilic/hydrophobic properties in water in response to temperature. The method was used to determine five sulfonylurea and three urea herbicides. Separation was achieved with a 10 mM ammonium acetate (pH 3.0) isocratic aqueous mobile phase, and by changing the column temperature. The analytes were extracted from water by off-line solid-phase extraction (SPE) with an *N*-vinyl-pyrrolidone polymer cartridge. The average recoveries of the eight herbicides from spiked pure water, tap water and river water were 70–130% with relative standard deviations (RSDs) of <10%. The limits of quantitation (LOQ) of the eight herbicides were between 1 and 4 $\mu g l^{-1}$.

Keywords: Poly(*N*-isopropylacrylamide); LC; Temperature-responsive chromatography; Sulfonylurea herbicides; Urea herbicides

1. Introduction

Herbicides are used in rice paddies, golf courses, and other types of fields. They are transported by aquifers in groundwater and are widely distributed in the environment. Sulfonylurea herbicides are labile, weakly acidic compounds. Sulfonylurea and urea herbicides are used at lower concentrations, and are more rapidly degraded in soil than older herbicides. Therefore, parts-per-billion concentrations of these herbicides are to be expected in the water supply. These herbicides have been analyzed in water by liquid chromatography (LC) with UV detection [1,2], capillary electrophoresis with UV [3], LC with mass spectrometry (MS) [4,5], immunoassay [6], bioassay [7] and radio immunoassay [1].

Recently, various polymers have been developed which change their structure in response to surrounding conditions, such as the pH, electric field, and temperature. Such polymers have been widely utilized in drug delivery systems [8], cell culture dishes [9], cell sheets [10] and bioconjugates [11]. Poly(*N*-isopropylacrylamide) (PNIPAAm) is one of these; it exhibits a thermally reversible phase transition in response to temperature changes across a lower critical solution temperature (LCST) of 32 °C in aqueous solution [12]. In water, the polymer chains of PNIPAAm hydrate and expand below this LCST, while they dehydrate to form a compact conformation above it. We previously reported a considerable and reversible change in the hydrophilic/hydrophobic properties of PNIPAAm-grafted surfaces in response to a change in temperature. Taking advantage of this characteristic, we developed an LC column packed with PNIPAAm to selectively separate analytes by controlling the external column temperature [13–17].

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0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.01.100

Temperature-responsive chromatography is a method with little load on the environment, because no organic solvent is used in the mobile phase. Urea herbicides in environmental water have been widely studied by Hogenboom and coworkers [2,18,19] and very rapid analyses were made by using a single short column for both SPE and analytical separation. However, there are fewer reports on sulfonylurea herbicides [5]. The aim of this study was to achieve the separation of both groups of herbicides by temperature-responsive LC with an aqueous mobile phase.

2. Experimental

2.1. Chemicals

Analytical-grade standards of bensulfuron-methyl (99.7%), imazosulfuron (99.7%), pyrazosulfuron-ethyl (99.9%), halosulfuron-methyl (100%), siduron (98.9%), daimuron (100.0%) and diuron (100.0%) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Analytical-grade flazasulfuron (99.9%) was purchased from Hayashi Pure Chemical Industries, Osaka, Japan. The structures of these herbicides are shown in Fig. 1. *N*-isopropylacrylamide (NIPAAm) was kindly provided by KOHJIN, Tokyo, Japan and was purified by recrystallization from *n*-hexane. 3-mercaptopropionic acid (MPA), 2,2′-azobisisobutyronitrile (AIBN), *N,N*-dimethylformamide (DMF), ethyl acetate, 1,4-dioxane, *N,N′*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide, HPLC-grade tetrahydrofran (THF) and ammonium acetate were purchased from Wako Pure Chemical Industries. Aminopropyl silica beads (average diameter, 5 μm; pore size, 120 Å) were purchased from Nishio Industries, Tokyo, Japan. The pure water used for sample preparation and the LC mobile phase was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The synthesis of PNIPAAm and a modification of aminopropyl silica with the NIPAAm polymer were carried out by radical polymerization, as previously reported [13,20].

2.2. Temperature-responsive LC

A PNIPAAm-grafted silica beads were packed into a stainless-steel column (150 mm × 4.6 mm i.d.). LC was carried out on an Agilent 1100 series (Agilent, Waldbronn, Germany) instrument equipped with a UV detector and a Rheodyne Model 7750 injector. The column oven was a product of Shodex AO-30C (Showa Denko, Tokyo, Japan). The mobile phase was 10 mM ammonium acetate (pH 3.0). The thermoresponsive elution behavior of the herbicides was monitored at 240 nm at a flow rate of 1.0 ml min^{-1} at various temperatures. The injection volume was 20 μl.

2.3. Preparation of standard solutions

Stock solutions (1000 mg l^{-1}) of each analytical standard were prepared in THF. Next, working standard mixtures were prepared by diluting each herbicide stock solution with THF. These stock solutions were stored at 4 °C. Standard solutions

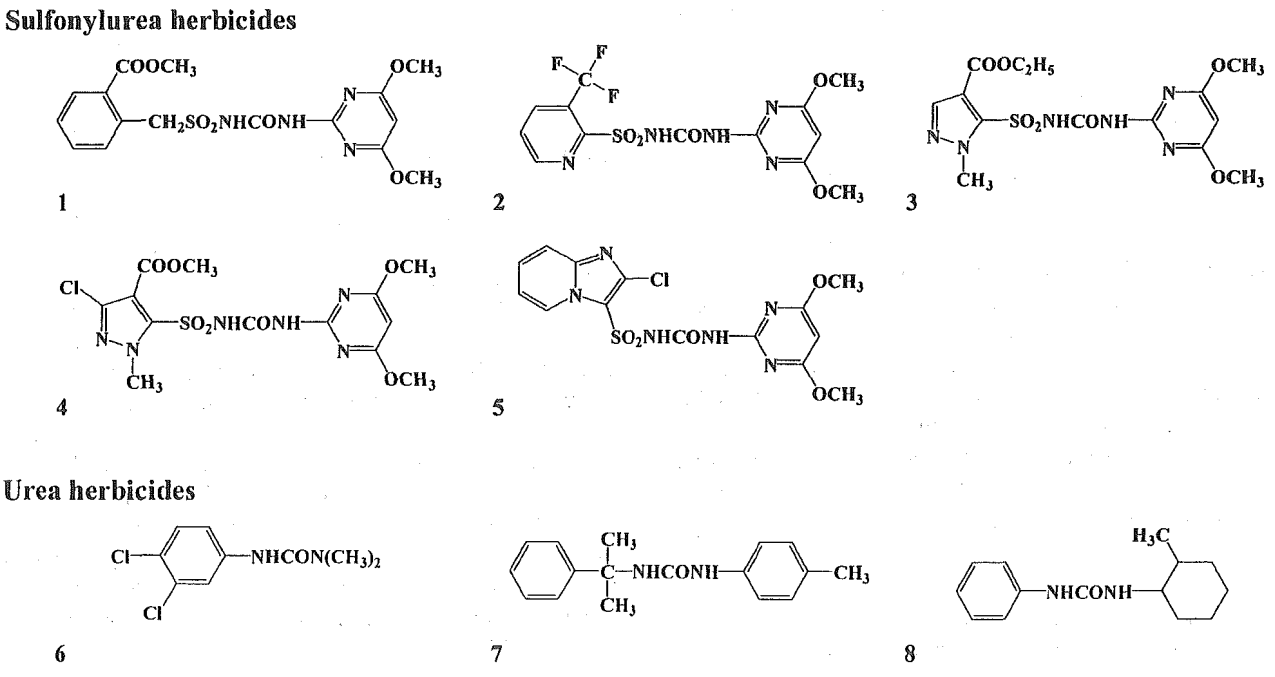

Fig. 1. Structures and common names of the eight herbicides. 1, bensulfuron-methyl; 2, flazasulfuron; 3, pyrazosulfuron-ethyl; 4, halosulfuron-methyl; 5, imazosulfuron; 6, diuron; 7, daimuron; and 8, siduron.

were prepared by diluting the stock solution with THF. The standard solutions were used for calibration plots and spiking of the water samples.

2.4. Water samples

Three types of water were analyzed: pure water, tap water and river water. The tap water was from a tap in the laboratory. L(+)-Ascorbic acid sodium salt (Wako Pure Chemical Industries) was added to the tap water at 0.005% (w/v), which eliminated chlorine that could react with and degrade some of the compounds of interest. The river water was collected from the Tama River near Tokyo; it was filtered through a glass-fiber filter before use.

2.5. Analytical methods

For recovery studies, three water samples (0.5 l each) were spiked with 1 ml of 2 mg l^{-1} (except for 0.5 mg l^{-1} diuron and daimuron) of the composite standard. Then, the spiked water samples were passed through a SPE cartridge to extract the analytes [5]. SPE was performed with cartridges prepacked with *N*-vinyl-pyrrolidone polymer resin (Oasis HLB Plus Extraction Cartridges) from Waters (Milford, MA, USA). The SPE cartridges were equilibrated with 5 ml of methanol and then 5 ml of pure water. The water samples were extracted at a 10 ml min^{-1} flow rate. Then, the cartridges were washed with 10 ml of pure water at a 5 ml min^{-1} flow rate and dried with air passed through the cartridge for 40 min. The herbicides were eluted from the cartridges with 3 ml of methanol at a speed of 1–2 drops s^{-1}. After evaporating the samples to near-dryness under a gentle nitrogen stream, the materials were dissolved to a final volume of 1.0 ml in THF.

3. Results and discussion

3.1. Sulfonylurea herbicides

Sulfonylurea herbicides were separated based on their temperature-controlled hydrophilic/hydrophobic properties by using an LC system connected to a column packed with PNIPAAm-modified silica beads. Fig. 2(a) shows van't Hoff plots for sulfonylurea herbicides separated using a PNIPAAm-modified column in 10 mM ammonium acetate (pH 3.0). The linearity in the van't Hoff plots is commonly observed for commercially available reversed-phase columns under standard chromatographic conditions. On the PNIPAAm-modified column, however, a deviation from linearity was found between ln *k* values and the reciprocal temperature (1/*T*). Interestingly, the slope of the van't Hoff plots of each analyte on the PNIPAAm-modified column changed markedly at the LCST boundary (Fig. 2 (a)). This corresponds to a phase transition of the polymer modified on the surface. Typical chromatograms for the standards of the five sulfonylurea herbicides using the PNIPAAm-modified column at 10 and 50 °C are shown in Fig. 3.

The log *P* values of these herbicides are given in Table 1. log *P* values were calculated by the CAChe system (Fujitsu, Japan). We reported in previous papers that the order of separation on a temperature-responsive-polymer-modified column depends on the hydrophobicities, corresponding to increasing log *P* values [13]. In this study, the retention time of the strongly hydrophobic imazosulfuron was remarkably increased, compared with four other sulfonylurea herbicides. When trying to separate the same herbicides on an ODS column using an aqueous/organic solvent, the three peaks of bensulfuron-methyl, flazasulfuron and imazosulfuron overlapped, and the two peaks of pyrazosulfuron-ethyl

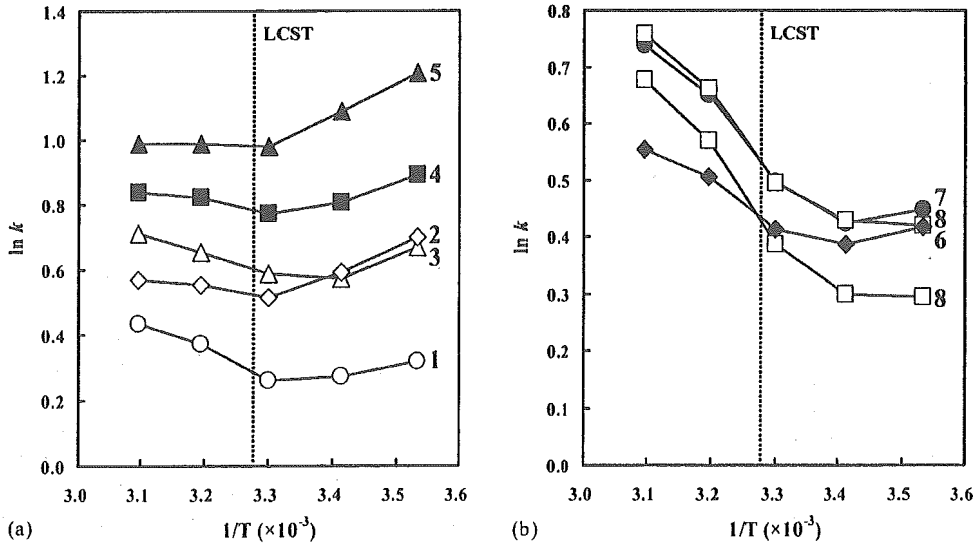

Fig. 2. van't Hoff plots of (a) sulfonylurea and (b) urea herbicides. For LC conditions, see Section 2. For peak numbers, see Fig. 1.

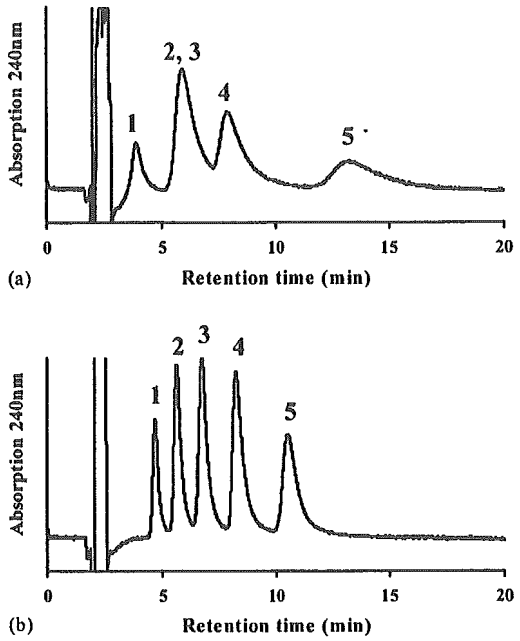

Fig. 3. LC–UV of standards using a PNIPAAm-modified silica column at (a) 10 °C and (b) 50 °C. For LC conditions, see Section 2. For peak numbers, see Fig. 1.

and halosulfuron-methyl also overlapped (data not shown). In contrast, upon raising the column temperature of the temperature-responsive system, these five sulfonylurea herbicides could be separated from each other with an aqueous mobile phase.

In this study, the mobile phase was adjusted to pH 3 which was lower than the pK_a values of these herbicides, bensulfuron-methyl (pK_a 5.2), flazasulfuron (pK_a 4.37) and imazosulfuron (pK_a 4.0), in order to suppress their ionization and effect their interaction with the surface of the stationary phase. With increasing temperature, the temperature-responsive surface of the stationary phase changed from hydrophilic to hydrophobic, the retention time increased as a result of hydrophobic interaction, and the separation of the five sulfonylurea herbicides markedly improved.

Table 1
Calibration, LOD and log P data for the eight herbicides

Compound	Calibration equation[a]	R^2	LOD (mg l^{-1})	log P
Bensulfuron-methyl	$y=12.493x+0.6557$	1.000	0.5	1.49
Flazasulfuron	$y=9.8272x-0.5951$	0.998	0.5	1.93
Pyrazosulfuron-ethyl	$y=8.976x-1.1398$	0.997	0.5	0.66
Halosulfuron-methyl	$y=12.011x-1.3876$	0.998	0.5	1.21
Imazosulfuron	$y=16.043x-0.951$	1.000	0.5	2.15
Diuron	$y=20.209x+0.6761$	0.996	0.5	2.15
Daimuron	$y=11.74x-0.2518$	0.995	0.2	3.61
Siduron	$y=13.661x-0.2925$	0.999	0.2	2.86

[a] y = area; x = concentration (mg l^{-1}).

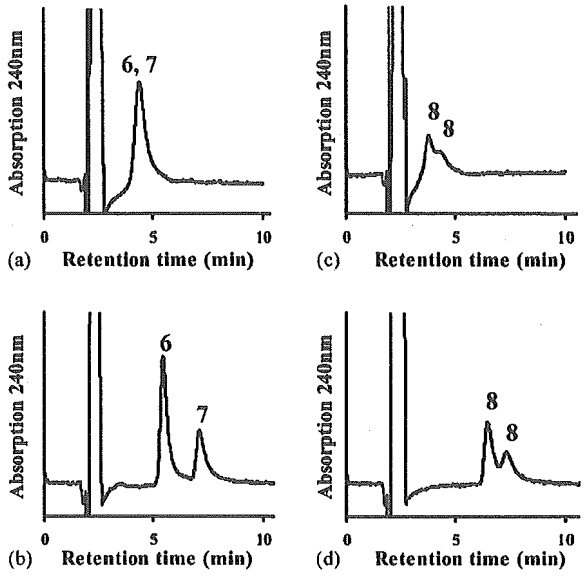

Fig. 4. LC–UV of standards using PNIPAAm-modified silica column at (a) and (c) 10 °C, and (b) and (d) 50 °C. For LC conditions, see Section 2. For peak numbers, see Fig. 1.

3.2. Urea herbicides

The urea herbicides were separated using conditions similar to those for the sulfonyulurea herbicides. Fig. 2(b) shows van't Hoff plots for urea herbicides using a PNIPAAm-modified column. For urea herbicides, the ln k values increased markedly above the LCST (or lower $1/T$ values), indicating a hydrophobic interaction between the analyte molecules and the hydrophobized stationary phase surface of the column. The difference in retention behavior of the sulfonylurea and urea herbicides reflects differences in their physicochemical properties. Typical chromatograms for the standards of the two urea herbicides, and siduron using the PNIPAAm-modified column at 10 and 50 °C are shown in Fig. 4. Siduron gave two peaks corresponding to its cis/trans isomers. The retention times of urea herbicides also increased with the log P values. An increase in the retention times with increasing temperature was clearly observed.

3.3. Analytical performance

The calibration plots of all eight herbicides using temperature-responsive LC at 50 °C were linear. The concentrations range of the five sulfonylurea herbicides were 0.2–10 mg l^{-1} (six data points in triplicate), those of diuron and daimuron were 0.2–2.0 mg l^{-1} (four data points in triplicate), and those of siduron were 0.5–10.0 mg l^{-1} (five data points in triplicate). In all cases, the R^2 values were at least 0.995 (Table 1). Because siduron has two isomers, the area of the two isomer peaks was calculated and summed to give the total amount of siduron. The LODs of the eight herbicides were 0.2–0.5 mg l^{-1} (Table 1).

Table 2
Performance data for extracting five sulfonylureas and three ureas from pure water, tap water and river water

Compound	Pure water			Tap water			River water		
	Recovery[a] (%)	RSD (%)	LOQ ($\mu g l^{-1}$)	Recovery[a] (%)	RSD (%)	LOQ ($\mu g l^{-1}$)	Recovery[a] (%)	RSD (%)	LOQ ($\mu g l^{-1}$)
Bensulfuron-methyl	91	3.6	4	94	2.2	1	88	6.4	4
Flazasulfuron	90	1.9	1	86	1.7	1	72	9.7	4
Pyrazosulfuron-ethyl	93	1.6	1	98	2.5	1	100	5.0	4
Halosulfuron-methyl	90	2.7	1	98	1.1	1	97	4.5	4
Imazosulfuron	86	1.8	1	98	1.8	1	89	6.7	4
Diuron	91	4.5	1	84	6.8	1	97	4.5	1
Daimuron	127	2.8	1	100	5.3	1	94	6.0	1
Siduron	93	2.5	1	87	3.2	4	100	6.0	4

[a] Mean values from three individual samples.

3.4. Application

Water samples were prepared by adding 4 $\mu g l^{-1}$ (final concentration) of all herbicides, except for diuron and daimuron, which were added at a final concentration of 1 $\mu g l^{-1}$ to pure water, tap water, or river water. Then, 0.5 l of each sample was concentrated 500-fold by SPE. Using temperature-responsive chromatography, these eight herbicides were detected with acceptable recoveries and precisions (70–130% and relative standard deviation, RSD $\leq$10%, respectively) (Table 2).

4. Conclusions

Temperature-responsive LC with an aqueous solution without organic solvents as mobile phase can be used to determine sulfonylurea and urea herbicides. Combined with off-line SPE, trace levels of the herbicide can be quantified in real-life samples.

In temperature-responsive LC, analyte behavior is controlled merely by the temperature, without any changes in the mobile-phase composition.

Acknowledgement

This study was supported by a grant from the Ministry of Health, Labour and Welfare of Japan.

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Use of cholinesterase activity as an indicator for the effects of combinations of organophosphorus pesticides in water from environmental sources

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Received 24 February 2005; received in revised form 9 August 2005; accepted 22 September 2005

Abstract

Organophosphorus pesticides (OPs) are commonly detected in agricultural products, animal-derived foodstuffs, and environmental samples. Until now, the focus of research has been to evaluate the adverse effect of a single OP. While each OP may be present at concentrations under recognized as "no observed adverse effect level (NOAEL)", the combined effects of multiple OPs present at these low concentrations have not been sufficiently studied. Therefore, we developed an in vitro testing method to evaluate the toxicity of multiple OPs based on the degree of inhibition of cholinesterase (ChE) activity. This method requires only 10 min to complete and no specialized technology. We examined 15 OPs by this method and categorized them into three groups according to the degree of ChE inhibition. A relationship between the OPs' chemical structures and the degree of ChE inhibition emerged with the moiety –P–O–C=N– showing the strongest action. The degree of ChE inhibition increased with multiple OPs, and the degree of inhibition seemed to be additive. These results demonstrate that the combined toxicity of multiple OPs present in food or environmental samples is an easily determined and toxicologically relevant measure of overall toxicity of complex OPs mixtures. It is possible to apply this testing method as a monitoring technique in water quality management in order to control OPs. As a result, this method can play the role for the potential risk reduction to the ecosystem and may contribute to the preservation of the environment.

Keywords: Organophosphorus pesticides; Hazard assessment; Cholinesterase activity; In vitro method; Combined toxicity; Additive action

1. Introduction

Recently, various environmental pollutants have become a major source of concern with regards to their potential adverse effects on humans exposed to combinations of these environmental pollutants. Water influences the health of both humans and animals and is greatly affected by water pollutants such as he organic compounds discharged from industrial, agricultural, and domestic drainage. Among the organic compounds, most concern, due to their

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0043-1354/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2005.09.042