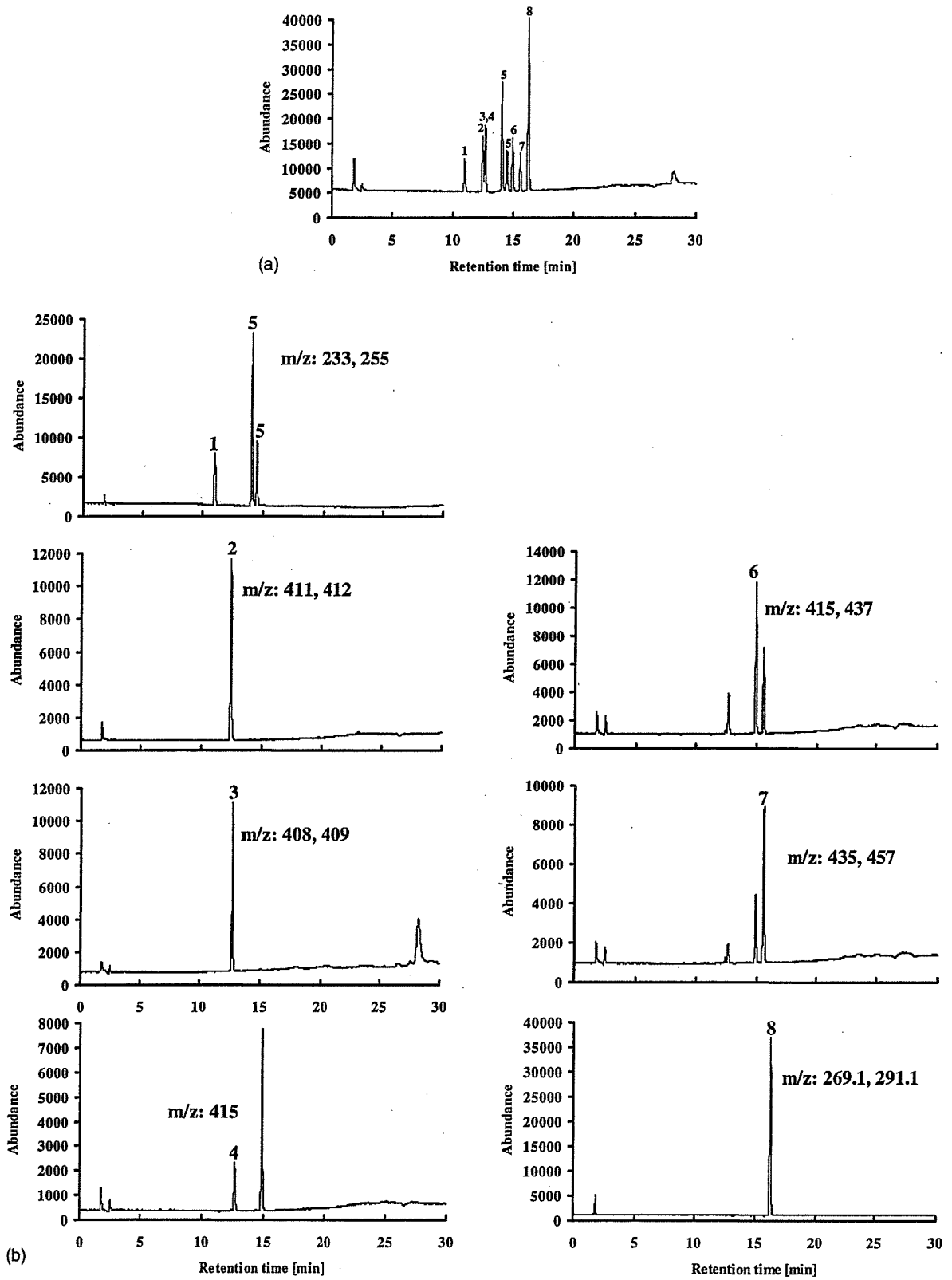

Fig. 2. (a) Chromatogram of a standard solution by LC–ESI–MS using selected ion monitoring (SIM). (b) Signals for each ion. The concentration of each compound was 0.02 mg l^{-1} in acetonitrile:water = 1:9 (v/v). Peaks—1: diuron (DCMU); 2: bensulfuron-methyl; 3: flazasulfuron; 4: imazosulfuron; 5: siduron; 6: pyrazosulfuron-ethyl; 7: halosulfuron-methyl; 8: dymron.

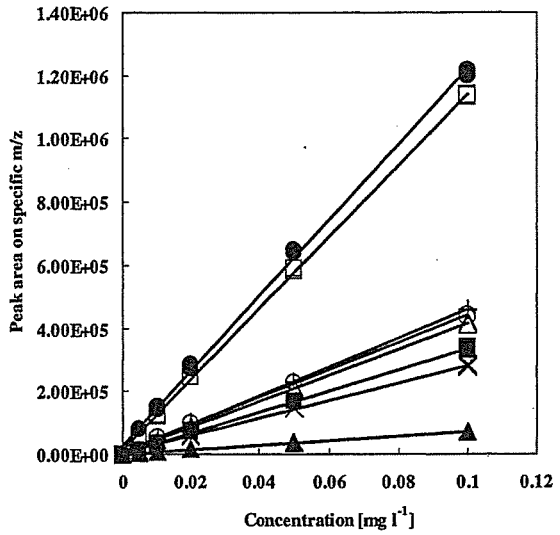

Fig. 3. Calibration curves of eight herbicides analyzed by LC–ESI–MS, with detailed signals shown for each ion. (○) Bensulfuron-methyl; (▲) imazosulfuron; (△) pyrazosulfuron-ethyl; (+) flazasulfuron; (■) halosulfuron-methyl; (□) siduron; (●) dymron; (×) diuron (DCMU).

dyne Model 7750 injector. The analytical column was a Zorbax Eclipse XDB-C_{18} (Agilent) 250 mm × 4.6 mm, 5 μm particle size. The mobile phases were 0.15% (v/v) acetic acid (A) and 100% acetonitrile (B). The herbicides were separated with the following gradient program: maintaining 60% A for 5 min; followed by a linear gradient from 60% A at $t = 5$ min to 20% A at $t = 20$ min; maintaining 20% A for 10 min and returning linearly to 60% A in 7 min. These LC conditions were described by Rodriguez and Orescan [18]. The column temperature was 40 °C, the flow-rate was 1.0 ml min^{-1} and the injection volume was 25 μl.

The MS system was an Agilent 1100 series (Agilent) quadrupole equipped with an electrospray ionization (ESI) source. The instrument was operated in the positive ionization mode. The operating conditions for ESI were nebulizer gas (nitrogen) 414 KPa; drying gas (nitrogen) flow 10.0 l min^{-1}; capillary voltage 4000 V and gas temperature 350 °C. The fragmentor voltage was kept at 120 V.

Table 2
Linear regression data for the eight herbicides investigated by LC–ESI–MS [calibration range 0, 0.005–0.1 mg l^{-1} (six data points)]

Compound	$y = bx + c$	r^2	LOD[a] (mg l^{-1})
Bensulfuron-methyl	$y = 4 \times E10^6\ x + 7688.7$	0.9992	0.005
Imazosulfuron	$y = 701937\ x + 1441.4$	0.9982	0.005
Pyrazosulfuron-ethyl	$y = 4 \times E10^6\ x + 4243.2$	0.9997	0.005
Flazasulfuron	$y = 5 \times E10^6\ x + 4884.8$	0.9996	0.005
Halosulfuron-methyl	$y = 3 \times E10^6\ x + 1447.6$	0.9997	0.005
Siduron	$y = 1 \times E10^7\ x + 11506$	0.9996	0.005
Dymron	$y = 1 \times E10^7\ x + 27683$	0.9984	0.005
Diuron (DCMU)	$y = 3 \times E10^6\ x + 2438$	0.9995	0.005

[a] LODs were evaluated by using a signal/noise (3) ratio of more than 3.

2.3. Preparation of standard solutions

Individual stock solutions (100 mg l^{-1}) of each analytical standard were prepared in acetonitrile. Next, the analytical standard mixtures were prepared by diluting each herbicide stock solution with acetonitrile to a final concentration of 12.5 mg l^{-1}. These stock solutions were stored at 4 °C. The 12.5 mg l^{-1} standard stock solution was appropriately diluted to prepare of five working standards; 0.005, 0.01, 0.02,

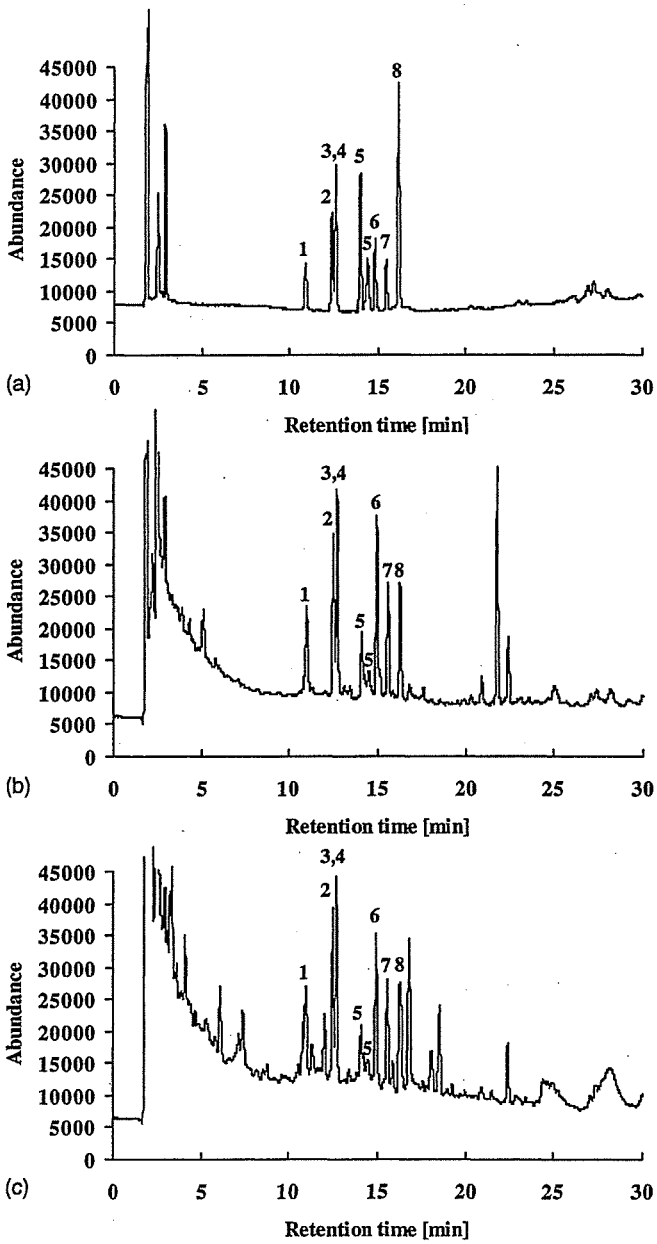

Fig. 4. Chromatogram of water sample fortified with the eight herbicides, using the LC–ESI–MS selected ion monitoring (SIM). (a) 40 ng l^{-1} in fortified pure water, (b) 100 ng l^{-1} fortified tap water (except for siduron and dymron, which were fortified to 20 ng l^{-1}) and (c) 100 ng l^{-1} fortified river water (except for siduron and dymron, which were fortified to 20 ng l^{-1}) Eight herbicides were concentrated by Oasis. Peaks—1: diuron (DCMU); 2: bensulfuron-methyl; 3: flazasulfuron; 4: imazosulfuron; 5: siduron; 6: pyrazosulfuron-ethyl; 7: halosulfuron-methyl; 8: dymron.

Table 3
Average recoveries, relative standard deviations (precision) and limits of quantitation (LOQs) on extracting five sulfonylureas and three ureas from pure water

Compound		20 ng l^{-1} [a]			40 ng l^{-1} [a]		
		Recovery (%)[b]	R.S.D. (%)	LOQ (ng l^{-1})	Recovery (%)[b]	R.S.D. (%)	LOQ (ng l^{-1})
Bensulfuron-methyl	PS2	93.8	1.5	2.8	106.8	0.8	3.3
	C_{18}	99.0	0.6	1.2	104.9	1.6	6.5
	Oasis	110.6	2.6	5.7	98.4	1.4	5.5
Imazosulfuron	PS2	81.4	0.4	0.6	105.0	3.0	12.5
	C_{18}	95.8	5.0	9.6	108.8	2.3	10.1
	Oasis	95.4	0.3	0.6	93.0	2.9	10.8
Pyrazosulfuron-ethyl	PS2	45.2	4.0	n.d.	72.8	1.3	3.7
	C_{18}	92.5	1.6	2.9	98.5	0.4	1.7
	Oasis	108.5	0.3	0.6	91.9	1.7	6.2
Flazasulfuron	PS2	31.9	1.8	n.d.	65.3	1.0	n.d.
	C_{18}	65.2	1.2	n.d.	72.3	0.2	0.7
	Oasis	90.2	1.6	2.9	89.1	0.4	1.5
Halosulfuron-methyl	PS2	70.9	2.8	3.9	108.6	3.5	15.3
	C_{18}	95.7	5.7	11.0	103.1	1.2	4.8
	Oasis	104.7	2.4	5.1	96.2	3.2	12.3
Siduron	PS2	102.8	1.4	2.8	113.5	0.8	3.7
	C_{18}	96.7	0.6	1.1	105.6	0.4	1.8
	Oasis	125.5	2.2	n.d.	111.7	1.1	4.7
Dymron	PS2	105.1	1.8	3.8	119.7	1.7	8.3
	C_{18}	101.0	2.1	4.3	115.8	1.0	4.8
	Oasis	109.8	3.7	8.1	107.3	0.5	2.3
Diuron (DCMU)	PS2	88.1	2.1	3.8	94.9	0.5	2.0
	C_{18}	88.6	2.9	5.1	94.8	0.3	1.1
	Oasis	87.7	2.7	4.7	91.0	0.9	3.2

n.d.: not determined.
[a] Fortified with pure water.
[b] Mean values from three determination.

0.05, and 0.10 mg l^{-1}. These were then used to analyze the fortified samples. The final solvent composition of the working standard solutions was 10:90 (acetonitrile:water).

2.4. Calibration procedure

The external standard method of calibration was used for this analysis. At least five standard solutions containing all eight compounds were analyzed, and calibration plots of the peak area as a function of the concentrations of analytes injected were linear over the range of 0, 0.005–0.10 mg l^{-1} in an automated sequence. Calibration curves were analyzed by LC–ESI–MS in selected ion mode (SIM mode), followed by detection of the signal from one or two of the more abundant daughter ions. These daughter ions were identified in scan mode during the acquisition of the mass spectrum of the selected ion. The injection was performed three times for each sample to test reproducibility.

2.5. Water samples

In this report, three types of water were analyzed: pure water, tap water and river water. The "pure" water was Milli-Q water. The tap water samples were collected from the tap in the laboratory. L(+)-ascorbic acid sodium salt (Wako) was added to tap water to 0.005% (w/v), and this eliminated the chlorine, which can react with and degrade some of the compounds of interest. River water samples were collected from the Tama river near Tokyo. The river water was filtered before use.

2.6. Analytical procedure

For recovery studies, three types of water samples (0.5 l of pure water, tap water and river water) were fortified with 1 ml of the composite standard solution. Afterwards, the fortified water samples were concentrated with the solid phase extraction (SPE) method as described previously [29]. The SPE was performed with PS2 cartridges that were prepacked with polystyrene polymer resin (Sep-Pak Plus PS2 Cartridges; PS2), C_{18} cartridges prepacked with ODS C_{18}-bonded silica resin (Sep-Pak Plus C_{18} Cartridges; C_{18}), and Oasis cartridges prepacked with *N*-vinyl-pyrrolidone polymer resin (Oasis HLB Plus Extraction Cartridges), all from Waters (Milford, MA, USA). The SPE mode was used for the pure water, tap water and river water samples to preconcentrate

a mixture of five sulfonylureas and three ureas. 0.5 l of each sample was extracted on the SPE cartridges packed with PS2, C_{18} and Oasis. Solid-phase cartridges were equilibrated with 5 ml acetonitrile (except OASIS which was conditioned with 5 ml methanol) then with 5 ml deionized water (Milli-Q water). Extraction of water samples was carried out at a 10 ml min^{-1} flow rate. After passing the sample through the cartridges, the cartridges were washed with 10 ml deionized water at a 5 ml min^{-1} flow rate. Air was then pulled through the cartridge for 40 min. The analytes were eluted from the cartridges with 3 ml acetonitrile (except Oasis, where 3 ml methanol was used) at a rate of 1–2 drops s^{-1}. After evaporating the samples to near dryness under a gentle nitrogen stream, the compounds were transferred into a final volume of 1.0 ml of a mixture of acetonitrile–water (ratio10:90).

3. Results and discussion

3.1. LC–ESI–MS analysis

The LC–ESI–MS system was used for quantitative analysis of water samples. The use of high-flow pneumatically assisted ESI run in positive mode is a soft ionization technique used for LC–MS applications. It is a superior interface for analysis of sulfonylurea and urea herbicides. In the present study, all of the herbicides chosen for investigation could be picked up by this detector. Selection of one or two ions for investigation was scheduled according to the following protocols, as detailed in Table 1. A typical chromatogram for the 0.02 mg l^{-1} standard solution containing the eight target analytes is shown in Fig. 2a. Although five of the eight herbicides were well separated, bensulfuron-methyl, imazosulfuron and flazasulfuron were not well separated in this chromatogram. Five sulfonylurea and three urea herbicides were detected by LC–ESI–MS in the selected ion monitoring (SIM). This was followed by extraction of the signal from one or two of the most abundant daughter ions. These were acquired in full scan mode during the acquisition of the mass spectrum of the selected ion. The ions used for SIM for each compound are summarized in Table 1. Each ion gave a strong ion signal with positive mode ESI, but imazosulfuron gave only a weak ion signal. Because bensulfuron-methyl will also be detected by *m/z* 413, which indicates the strong ion of imazosulfuron, the peaks of these compounds overlap, and it is difficult to quantitate each of

Table 4
Average recoveries, relative standard deviations (precision) and limits of quantitation (LOQs) on extracting five sulfonylureas and three ureas from tap and river water

Compound		Tap water (0.5 l)[a]			River water (0.5 l)[a]		
		Recovery (%)[b]	R.S.D. (%)	LOQ (ng l^{-1})	Recovery (%)[b]	R.S.D. (%)	LOQ (ng l^{-1})
Bensulfuron-methyl	PS2	39.2	1.0	n.d.	95.9	0.8	7.8
	C_{18}	72.2	1.1	7.8	64.3	0.6	n.d.
	Oasis	90.8	1.0	9.2	93.2	0.7	6.7
Imazosulfuron	PS2	61.4	3.1	n.d.	98.8	1.6	15.7
	C_{18}	73.8	5.3	38.3	75.6	0.9	6.6
	Oasis	88.6	0.5	4.5	82.7	3.4	27.7
Pyrazosulfuron-ethyl	PS2	47.8	5.0	n.d.	45.3	1.6	n.d.
	C_{18}	20.8	2.1	n.d.	9.1	2.1	n.d.
	Oasis	112.9	1.6	18.1	86.4	0.6	5.0
Flazasulfuron	PS2	33.3	1.5	n.d.	51.5	0.5	n.d.
	C_{18}	28.8	0.3	n.d.	29.2	0.7	n.d.
	Oasis	100.2	0.9	8.9	98.6	0.4	3.8
Halosulfuron-methyl	PS2	55.9	9.2	n.d.	63.2	11.4	n.d.
	C_{18}	27.5	1.6	n.d.	16.1	3.0	n.d.
	Oasis	81.4	2.5	20.4	81.8	3.0	24.5
Siduron	PS2	98.5	2.7	5.2	104.5	3.2	6.7
	C_{18}	95.9	1.5	2.9	95.0	1.6	3.1
	Oasis	108.1	3.7	8.0	110.2	0.6	1.4
Dymron	PS2	116.4	2.7	6.3	94.5	0.6	1.2
	C_{18}	112.2	2.8	6.4	89.3	3.6	6.5
	Oasis	108.0	1.6	3.6	80.9	1.2	2.0
Diuron (DCMU)	PS2	123.6	2.2	n.d.	124.8	2.2	n.d.
	C_{18}	131.1	1.1	n.d.	133.5	3.3	n.d.
	Oasis	115.6	0.8	9.3	107.2	6.1	65.7

n.d.: not determined.
[a] Fortified with 100 ng l^{-1}, except for siduron and dymron fortified with 20 ng l^{-1}.
[b] Mean values from three determination.

the compounds. Therefore, a peak found at *m/z* 415 was chosen for detection, even though the area under this peak was smaller, because it was possible to get better quantitative determinations without complicating peak overlaps. The compound siduron gave two peaks, because it had two isomeric forms. The area under the two peaks was calculated and summed to give the total amount of siduron in the samples. Fig. 2b shows an extracted ion chromatogram for a standard solution.

The concentration of acetic acid in mobile phase will change during the gradient elution. However, the separation behavior is dependent on the affinity on the surface of the solid phase according to the concentration of the organic solvent in mobile phase. Therefore, the change of the concentration of acetic acid might not become a problem for the analysis of the herbicides shown in this paper. The quantitation and reproducibility have been obtained under this method.

3.2. Calibration curves and limit of detection (LOD)

Calibration curves were established for the eight substances, each of which was analyzed at five different concentrations. Standard solutions containing 0, 0.005, 0.01, 0.02, 0.05, and 0.10 mg l^{-1} were analyzed by LC–ESI–MS in the SIM mode. Examples of calibration curves for the eight analytes are shown in Fig. 3. Linear regression data are shown in Table 2. The calibration curves were linear in the concentration range from 0 to 0.10 mg l^{-1} and the correlation coefficients were higher than 0.998 for all of the herbicides studied. This indicated that the methodology developed in this work performed well in quantitating these compounds.

The LODs were defined as three-times of standard deviation (S.D.) on the basis of three independent determinations. The LODs can be obtained using this methodology due to the high selectivity and sensitivity of the LC–MS system. The LODs using this method are 0.005 mg l^{-1} in standard solution for all herbicides.

3.3. Limit of quantitation (LOQ)

This method was validated using pure water, tap water, and river water. The standard mixture solution was added to pure water, tap water or river water, respectively, and the recovery by three kinds of solid phases, i.e. PS2, C_{18}, and Oasis, was examined using each 0.5 l sample. Water samples were fortified with five selected sulfonylurea and three selected urea herbicides. Water samples with 20 and 100 ng l^{-1} of each compound were prepared. Pure water samples were fortified with both 20 and 40 ng l^{-1} of each compound, while tap water and river water were fortified with 100 ng l^{-1} of each compound, with the exception of siduron or dymron, which were added to a final concentration of 20 ng l^{-1}. All samples were concentrated to 500-fold. Typical chromatograms of fortified pure water, tap water and river water samples are shown in Fig. 4. Average recoveries and relative standard deviations (R.S.D.s) are summarized in Tables 3 and 4.

LOQ was the lowest fortification level evaluated. The methods were used for determining with acceptable recoveries and precisions (70–120% and R.S.D. $\leq$ 20%, respectively). The LOQ is defined as the concentration that is 10-times the value S.D. on the basis of three independent determinations. The results of herbicide measurements are shown in Table 3 and Fig. 5 (fortified pure water) and Table 4 and Fig. 6 (fortified tap water and river water). The recoveries were between 70 and 120% and the R.S.D.s were less than 20%. It shows that n.d. does not satisfy these two conditions. In this investigation, the LOQ for the fortified waters was 10 ng l^{-1} for the seven compounds (except halosulfuron-methyl was 20 ng l^{-1}) in the pure water, using any of the three kinds of cartridge. Similarly, the LOQ in the case of tap water was 10 ng l^{-1} (except imazosulfuron, pyrazosulfuron-ethyl and halosulfuron-methyl, which had the LOQ of 100 ng l^{-1}), for river water the LOQ was 10 ng l^{-1} (except imazosulfuron, halosulfuron-methyl and diuron was 100 ng l^{-1}).

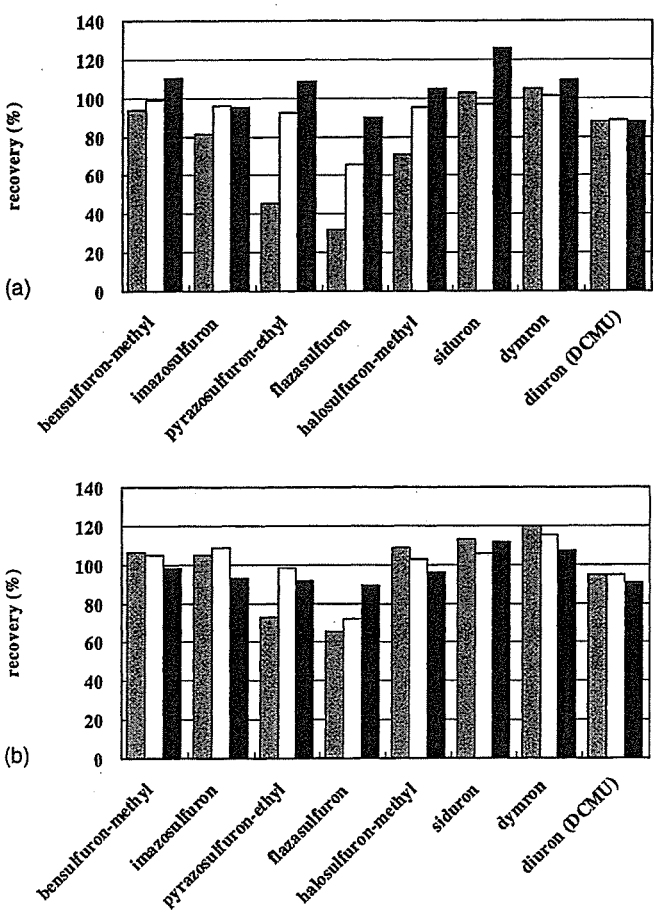

Fig. 5. Levels of herbicide recovery of from pure water, using PS2, C_{18} and Oasis cartridges. (a) Fortified with 20 ng l^{-1}; (b) fortified with 40 ng l^{-1}. (▨): PS2; (□): C_{18}; (■): Oasis.

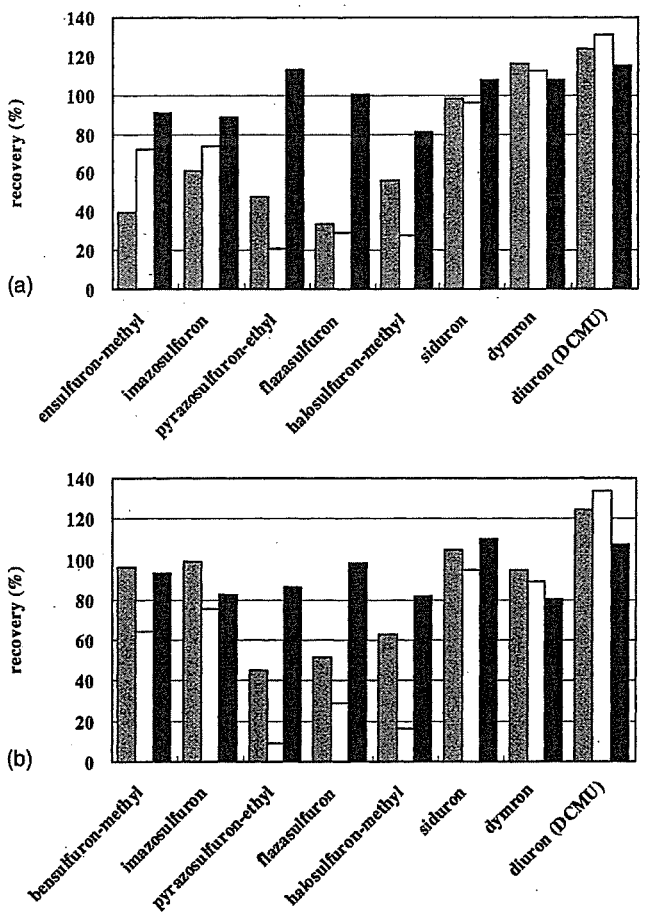

Fig. 6. Levels of herbicide recovery from water samples using PS2, C_{18} and Oasis cartridges. (a) Tap water; (b) river water. (▨): PS2; (□): C_{18}; (■): Oasis.

4. Conclusions

We have demonstrated that SPE–LC–ESI–MS is a sensitive and selective technique for the determination and quantitation of herbicides in environmental water samples. Very low detection limits can be reached due to the enhanced selectivity and high sensitivity obtained with this methodology. Furthermore, this method has clearly demonstrated good recoveries (70–120%), good precision (0.2% ≤ R.S.D. ≤ 5.7%), and good sensitivity by SPE using the three different types of cartridge.

Acknowledgements

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Early Pregnancy Failure Induced by Dibutyltin Dichloride in Mice

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ABSTRACT: In this study, we examined the adverse effects of dibutyltin on initiation and maintenance of pregnancy after maternal administration during early pregnancy in mice. Following successful mating, female ICR mice were given dibutyltin dichloride (DBTCl) at 0, 7.6, 15.2, or 30.4 mg/kg bw/day by gastric intubation on days 0–3 or days 4–7 of pregnancy. Female mice were sacrificed on day 18 of pregnancy, and the pregnancy outcome was determined. After administration of DBTCl on days 0–3, the rate of non-pregnant females and the incidence of preimplantation embryonic loss were significantly increased at 30.4 mg/kg bw/day. The incidences of postimplantation embryonic loss in females given DBTCl on days 0–3 at 15.2 mg/kg and higher and on days 4–7 at 7.6 mg/kg bw/day and higher were increased. No increase in the incidence of fetuses with external malformations was observed after the administration of DBTCl on days 0–3 or days 4–7. A decline in the serum progesterone levels was detected in mice given DBTCl at 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The data show that DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice and suggest that the decline in serum progesterone levels is responsible for pregnancy failure. © 2007 Wiley Periodicals, Inc. Environ Toxicol 22: 44–52, 2007.

Keywords: dibutyltin dichloride; organotin; pregnancy failure; early embryonic loss; progesterone

INTRODUCTION

Organotin compounds are chemicals widely used in agriculture and industry. Disubstituted organotin compounds are commercially the most important derivatives, being used as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during the melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers (Piver, 1973; WHO, 1980). Wide-spread use of organotin compounds has caused increasing amounts to be released into environment. The most important route of entry of organotin compounds as nonpesticides into the environment is through the leaching of organotin-stabilized PVC by water (Quevauviller et al., 1991), and its use in antifouling agents resulting in the entry of organotin into the aquatic environment (Maguire, 1991). The identification of dibutyltin (DBT) and tributyltin (TBT) in aquatic marine organisms (Sasaki et al., 1988; Lau, 1991) and marine products (Suzuki et al., 1992) has been reported. TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT in the environment (Seligman et al., 1988; Stewart and de Mora, 1990). Food chain bioaccumulation of butyltin in oysters (Waldock and Thain, 1983), mud crabs (Evans and Laughlin, 1984), marine mussels (Laughlin et al., 1986), Chinook salmon (Short and Thrower, 1986), and dolphin, tuna, and shark (Kannan et al., 1996) has been reported. These findings indicate that butyltins accumulate in the

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food chain and are bioconcentrated, and that humans can be exposed to butyltins via food.

Organotins possesses toxic effects on reproduction and development in experimental animals (Ema and Hirose, 2006). We previously reported that dibutyltin dichloride (DBTCl) by gavage throughout the period of organogenesis resulted in a significant increase in the incidence of fetal malformations in rats (Ema et al., 1991) and that rat embryos were highly susceptible to the teratogenic effects of DBTCl when administered on day 7 and day 8 of pregnancy (Ema et al., 1992). Tetrabutyltin (TeBT) is metabolized to TBT, DBT, and monobutyltin (MBT) derivatives (Fish et al., 1976; Kimmel et al., 1977). The TBT compound is metabolized to DBT and MBT derivatives and DBT is metabolized to MBT derivatives (Iwai et al., 1981). The developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from those of TeBT, TBT, and MBT in its mode of action, because the susceptible period for teratogenicity and types of malformations induced by DBT are different from those induced by TeBT, TBT, and MBT (Ema et al., 1995, 1996). Tributyltin chloride (TBTCl) (Harazono et al., 1996, 1998ab) and DBTCl (Ema and Harazono, 2000ab) during early pregnancy produced pregnancy failure in rats. In rats, the predominant adverse effects on reproduction and development of TBTCl and DBTCl on days 0–3 of pregnancy were a decrease in the pregnancy rate and an increase in the incidence of preimplantation embryonic loss, and TBTCl and DBTCl on days 4–7 of pregnancy mainly caused postimplantation embryonic loss (Harazono et al., 1998b; Ema and Harazono, 2000ab). The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl (Ema and Harazono, 2000b). Thus, the possibility exists that DBTCl and/or metabolites participate in the induction of early embryonic loss due to TBTCl.

The reproductive and developmental effects of organotin compounds, including DBT, were extensively investigated in rats (Ema and Hirose, 2006). We are unaware of any studies in which the adverse effects of DBT on initiation and maintenance of pregnancy have been assessed in mice. Studies in mice would be of great value in evaluating the reproductive and developmental toxicity of DBT. The present study was therefore conducted to determine the adverse effects on the initiation and maintenance of pregnancy of maternal exposure to DBTCl during early pregnancy in mice.

MATERIALS AND METHODS

Animal Husbandry and Maintenance

Male and female Crlj:CD1(ICR) mice at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, (Yokohama, Japan). The mice were acclimatized to the laboratory for 11 days prior to the start of the experiment. Male and female mice found to be in good health were selected for use. Female mice were caged with male mice and checked the following morning for signs of successful mating by examining vaginal plugs. The day when vaginal plugs were detected was considered to be day 0 of pregnancy. Successfully mated females were distributed into eight groups of 12 mice each and housed individually. Animals were reared on a γ-irradiated basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and filtered tap water *ad libitum*, and maintained in an air-conditioned room at 22°C ± 3°C, with a relative humidity of 50% ± 20%, under a controlled 12 h light/dark cycle, and ventilation with 10–15 air changes/hour. This study was performed in 2005 at the Safety Research Institute for Chemical Compounds. (Sapporo, Japan) in compliance with the "Law for the Humane Treatment and Management of Animals" (Ministry of the Environment, Japan, 1973), "Standards Relating to the Care and Management, etc. of Experimental Animals" (Prime Minister's Office, Japan, 1980) and "Guidance for Animal Care and Use of the Safety Research Institute for Chemical Compounds, Co."

Chemicals and Dosing

DBTCl was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The DBTCl used in this study was 99.5% pure, and it was kept in a dark and cool place. DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Osaka, Japan). The female mice were dosed once daily by gastric intubation with DBTCl at a dose of 7.6, 15.2, or 30.4 mg/kg bw (25, 50 or 100 μmol/kg bw) on days 0–3 of pregnancy or on days 4–7 of pregnancy. The dosage levels were determined based on the results of our previous studies, in which increases in the incidence of pre- and postimplantation embryonic loss were caused in female rats gavaged with DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 and days 4–7 of pregnancy, respectively (Ema and Harazono, 2000ab) and our dose-finding study in which no adverse effects on embryonic survival at 15.2 mg/kg bw/day and lower, increased embryonic loss at 30.4 mg/kg bw/day, and one death and three pregnancy failure in four females at 60.8 mg/kg bw/day were found in mice gavaged with DBTCl on days 0–3 of pregnancy. The volume of each dose was adjusted to 5 mL/kg of body weight based on the daily body weight. The control mice received olive oil only on days 0–3 or days 4–7 of pregnancy. All DBTCl solutions were prepared fresh daily.

Observations

All mice were observed for clinical signs of toxicity twice a day during the administration period and daily during the nonadministration period. Females showing a moribund condition were euthanized under ether anesthesia. Maternal

TABLE I. Maternal findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	1	0	0
Moribund condition (euthanized)	0	1	1	1
Vaginal discharge	0	1	0	0
Jaundice	0	2	7*	10*
Decreased locomotor activity	0	2	1	1
Hypothermia	0	1	1	1
Soil of perigenital fur	0	0	1	0
Initial body weight (g)[a]	27.4 ± 2.0	27.2 ± 2.1	27.2 ± 2.4	27.2 ± 2.1
Body weight gain (g)[a]				
Days 0–4	1.7 ± 1.1	0.6 ± 1.2	1.2 ± 1.6	0.3 ± 0.9*
Days 4–8	2.9 ± 1.5	2.5 ± 2.6	2.1 ± 2.0	1.6 ± 1.5
Days 8–18	20.1 ± 9.1	21.3 ± 12.4	13.6 ± 12.2	8.6 ± 12.2
Adjusted weight gain[b]	8.9 ± 3.4	9.9 ± 3.8	7.9 ± 4.8	5.3 ± 5.0
Food consumption (g)[a]				
Days 0–4	18.2 ± 1.8	15.0 ± 1.9*	16.7 ± 3.2	14.8 ± 2.3*
Days 4–8	22.9 ± 4.9	22.0 ± 2.7	21.7 ± 3.5	20.9 ± 3.5
Days 8–18	71.7 ± 10.1	71.0 ± 12.5	64.6 ± 13.3	57.8 ± 13.4*

[a]Values are given as mean ± SD.
[b]Adjusted weight gain refers to body weight gain excluding the uterus.
* Significantly different from the control, $P < 0.05$.

body weight was recorded daily, and food consumption was recorded on days 0, 4, 8, 12, and 18 of pregnancy. The females were euthanized by exsanguination under ether anesthesia on day 18 of pregnancy. The uterus was weighed and the number of corpora lutea was recorded. The numbers of implantations, live and dead fetuses, and of resorptions were counted. The uteri were placed in 10% ammonium sulfide for confirmation of the dam's pregnancy status (Salewski, 1964). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. The placental weight was also measured.

Analysis of Serum Steroids Hormone Levels

Blood samples were collected from the abdominal aorta under ether anesthesia on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl at 0 or 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The serum was separated and stored at −80°C for later assay of steroid hormones. Serum progesterone and 17β-estradiol were measured by Teizo Medical (Kawasaki, Japan) using the liquid chromatography-electrospray ionization Tandem Mass Spectrometry (LC-MS/MS, Applied Biosystems/MDS SCIEX). The detection limits of serum progesterone and 17β-estradiol were 10.0 and 0.25 pg/mL, respectively. The intra- and interassay coefficients of variation for 17β-estradiol were below 6.4% and 8.9%, respectively. The intra- and interassay coefficients of variation for progesterone were below 9.0% and 7.9%, respectively.

Statistical Analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, food consumption, numbers of corpora lutea, implantations, embryonic/fetal loss and live fetuses, fetal weight, and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney U test. The incidences of pre- and postimplantation embryonic loss and fetuses with external malformations were analyzed using Wilcoxon's rank sum test. The incidence of clinical signs in dams, pregnancy, nonpregnancy, and litters with fetal malformations, and the sex ratio of live fetuses were analyzed using Fisher's exact test. The levels of serum progesterone and 17β-estradiol were analyzed by Student's *t*-test. The 0.05 level of probability was used as the criterion for significance.

TABLE II. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	3	4	7*
No. of pregnant females	11	9	8	5*
No. of implantations per female[a,b]	9.5 ± 5.1	9.8 ± 7.1	8.3 ± 7.0	5.4 ± 6.7
Pre-implantation loss per female (%)[a,b]	9.7	29.7[c]	34.0	58.3*
No. of pregnant females surviving until scheduled sacrifice	11	8	7	4
No. of litters totally resorbed	0	0	1	1
No. of corpora lutea per litter[a,d]	10.5 ± 4.3	13.1 ± 4.9	12.4 ± 4.4	13.3 ± 1.3
No. of implantations per litter[a,d]	10.4 ± 4.3	12.6 ± 4.9	12.3 ± 4.4	13.3 ± 1.3
Pre-implantation loss per litter (%)[d,e]	1.5	3.3	1.1	0
No. of post-implantation loss per litter[a,d]	1.0 ± 1.0	1.1 ± 1.5	4.1 ± 3.2	4.0 ± 5.4
Post-implantation loss per litter (%)[d,f]	10.1	14.1	41.3*	32.2
No. of live fetuses per litter[a,d]	9.4 ± 4.2	11.5 ± 5.3	8.1 ± 5.0	9.3 ± 6.2
Sex ratio of live fetuses (male / female)	50/53	47/45	30/27	21/16
Body weight of live fetuses (g)[a]				
Male	1.54 ± 0.19	1.30 ± 0.12*	1.14 ± 0.22*	1.12 ± 0.10*
Female	1.42 ± 0.15	1.28 ± 0.20	1.08 ± 0.26*	1.01 ± 0.11*
External examinations of fetuses				
No. of fetuses (litters) examined	103 (11)	92 (8)	57 (6)	37 (3)
No. of fetuses (litters) with anomalies	1 (1)	0	1 (1)	0
Cleft palate	1	0	1	0
Kinked tail	0	0	1	0
Placental weight (mg)[a]	125 ± 56	116 ± 15	120 ± 17	119 ± 16

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

RESULTS

Administration of DBTCl on Days 0–3 of Pregnancy

Table I shows the maternal findings in mice given DBTCl on days 0–3 of pregnancy. One death was observed at 7.6 mg/kg bw/day, and one female each showed a moribund condition at 7.6, 15.2, and 30.4 mg/kg bw/day, and was euthanized. The female mice in the DBTCl-treated groups showed vagina discharge, jaundice, decreased locomotor activity, hypothermia and/or soiled perigenital fur, and the incidence of females showing jaundice was significantly increased at 15.2 mg/kg bw/day and higher. A significantly decreased body weight gain on days 0–4 was noted at 30.4 mg/kg bw/day. Food consumption on days 0–4, days 4–8, and days 8–18 in the DBTCl-treated groups were reduced, and significantly decreased food consumptions on days 0–4 at 7.6 and 30.4 mg/kg bw/day and on days 8–18 at 30.4 mg/kg bw/day were observed.

The reproductive and developmental findings in mice given DBTCl on days 0–3 of pregnancy are shown in Table II. The total absence of any implantation site, i.e., nonpregnancy, was found in one, three, four, and seven of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively. In the successfully mated females, the pregnancy rate was significantly decreased, and the incidence of preimplantation embryonic loss per females was significantly increased at 30.4 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, the number of corpora lutea per litter, implantations per litter, live fetuses per litter, the incidence of litters totally resorbed and of preimplantation loss per litter, and the sex ratio of live fetuses were not significantly different between the control and DBTCl-treated groups. The incidence of postimplantation loss per litter was increased in the DBTCl-treated groups, and a significant increase was observed at 15.2 mg/kg bw/day. A significantly lower fetal weight was found in males at 7.6 mg/kg bw/day and in both sexes at 15.2 and 30.4 mg/kg bw/day. One fetus with cleft palate in the control group and one fetus with a cleft palate and kinked tail in the 15.2 mg/kg bw/day group were observed. The placental weight in the DBTCl-treated

TABLE III. Maternal findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	0	1	0
Moribund condition (euthanized)	0	0	0	1
Vaginal discharge	0	0	4	4
Jaundice	0	0	2	6*
Decreased locomotor activity	0	0	0	1
Hypothermia	0	0	0	1
Initial body weight (g)[a]	28.1 ± 1.8	28.1 ± 1.8	28.1 ± 1.8	28.2 ± 1.7
Body weight gain (g)[a]				
Days 0–4	1.6 ± 1.0	1.9 ± 0.8	1.2 ± 1.2	1.6 ± 0.9
Days 4–8	3.1 ± 1.1	1.9 ± 1.6	0.5 ± 1.8*	−0.3 ± 2.1*
Days 8–18	24.9 ± 9.1	14.9 ± 8.9*	2.9 ± 6.3*	2.4 ± 2.4*
Adjusted weight gain[b]	8.3 ± 3.5	8.1 ± 4.3	3.2 ± 5.3*	3.8 ± 3.2*
Food consumption (g)[a]				
Days 0–4	18.5 ± 1.9	18.9 ± 2.4	18.4 ± 2.7	18.8 ± 1.3
Days 4–8	21.8 ± 1.9	19.2 ± 2.6	16.4 ± 3.3*	15.6 ± 3.5*
Days 8–18	74.5 ± 12.1	67.7 ± 9.9	55.2 ± 12.6*	57.2 ± 6.2*

[a] Values are given as mean ± SD.
[b] Adjusted weight gain refers to body weight gain excluding the uterus.
* Significantly different from the control, $P < 0.05$.

groups was not significantly different from that in the control group.

Administration of DBTCl on Days 4–7 of Pregnancy

Table III shows the maternal findings in mice given DBTCl on days 4–7 of pregnancy. One death was observed at 15.2 mg/kg bw/day, and one female that showed a moribund condition at 30.4 mg/kg bw/day was euthanized. The female mice in the DBTCl-treated groups showed vaginal discharge, jaundice, decreased locomotor activity, and/or hypothermia, and the incidence of females with jaundice was significantly increased at 30.4 mg/kg bw/day. The body weight gain on days 4–8 and adjusted weight gain, which indicates the net weight gain of female mice, at 15.2 mg/kg bw/day and higher, and on days 8–18 at 7.6 mg/kg bw/day and higher were significantly decreased. Food consumption on days 4–8 and days 8–18 was significantly lowered at 15.2 mg/kg bw/day and higher.

The reproductive and developmental findings in mice given DBTCl on days 4–7 of pregnancy are presented in Table IV. Although nonpregnancy was found in one, two, and one of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively, no significant decrease in the pregnancy rate was noted in the DBTCl-treated groups. In the successfully mated females, the number of implantations per female was significantly decreased at 15.2 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, totally resorbed litters were found in 2 of the 11 females at 7.6 mg/kg bw/day, 8 of the 9 females at 15.2 mg/kg bw/day, and 10 of the 10 females at 30.4 mg/kg bw/day. At 30.4 mg/kg bw/day, no live fetuses were obtained. The numbers of corpora lutea per litter, implantations per litter, and preimplantation loss per litter, and the sex ratio of live fetuses in the DBTCl-treated groups were not significantly different from those in the control group. A significant increase in the number and incidence of postimplantation loss per litter, and a decrease in the number of live fetuses were found in the DBTCl-treated groups. The weights of male and female fetuses were significantly lowered at 7.6 mg/kg bw/day. One fetus with omphalocele, and one fetus with exencephaly and open eyelids were observed at 7.6 mg/kg bw/day. The placental weight was not significantly different between the control and the DBTCl-treated groups.

Serum Progesterone and 17β-Estradiol Levels

The serum progesterone and 17β-estradiol levels are shown in Figure 1. A significant reduction in the serum progesterone levels was noted in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Although higher levels of serum 17β-estradiol were observed after the administration of DBTCl on days 4–7 of pregnancy, no statistically significant difference in 17β-estradiol levels were detected between the control and DBTCl-treated groups.

DISCUSSION

The present study was designed to evaluate the adverse effects of DBTCl on the initiation and maintenance of

TABLE IV. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	1	2	1
No. of pregnant females	11	11	10	11
No. of implantations per female[a,b]	12.6 ± 4.4	13.2 ± 4.6	7.5 ± 5.7*	11.1 ± 5.4
Pre-implantation loss per female (%)[a,b]	8.9	8.9	24.7	18.3[c]
No. of pregnant females surviving until scheduled sacrifice	11	11	9	10
No. of litters totally resorbed	0	2	8*	10*
No. of corpora lutea per litter[a,d]	13.8 ± 2.1	14.5 ± 2.3	10.6 ± 5.2	13.9 ± 2.8
No. of implantations per litter[a,d]	13.7 ± 2.1	14.4 ± 2.2	9.4 ± 5.1	12.7 ± 4.1
Pre-implantation loss per litter (%)[d,e]	0.6	0.6	10.7	10.2
No. of postimplantation loss per litter[a,d]	0.6 ± 1.0	7.2 ± 6.1*	8.7 ± 4.8*	12.7 ± 4.1*
Post-implantation loss per litter (%)[d,f]	4.3	48.3*	94.4*	100*
No. of live fetuses per litter[a,d]	13.1 ± 2.0	7.2 ± 5.6*	0.8 ± 2.3*	0
Sex ratio of live fetuses (male/female)	82/62	50/29	4/3	
Body weight of live fetuses (g)[a]				
Male	1.45 ± 0.10	1.23 ± 0.10*	1.27	
Female	1.39 ± 0.10	1.18 ± 0.14*	1.18	
External examinations of fetuses				
No. of fetuses (litters) examined	144 (11)	79 (9)	7 (1)	
No. of fetuses (litters) with anomalies	0	2 (2)	0	
Omphalocele	0	1	0	
Exencephaly and open eyelids	0	1	0	
Placental weight (mg)[a]	102 ± 10	99 ± 12	114	

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

pregnancy following maternal exposure during early pregnancy in mice. The most striking finding in the present study is pregnancy failure, decrease in the pregnancy rate, and litters totally resorbed, in females given DBTCl during early pregnancy.

Death and/or moribund condition were observed after the administration of DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 of pregnancy and at 15.2 mg/kg bw/day and higher on days 4–7 of pregnancy, and significant increased incidence of females showing clinical signs of toxicity were found after the administration of DBTCl at 15.2 mg/kg bw/day and higher on days 0–3 of pregnancy and at 30.4 mg/kg bw/day on days 4–7 of pregnancy. These findings indicate that more severe general toxicity was induced by DBTCl on days 0–3 of pregnancy than that on days 4–7 of pregnancy. However, adverse effects on body weight gain were detected after the administration of DBTCl at 30.4 mg/kg bw/day on days 0–3 of pregnancy and at 7.6 mg/kg bw/day and higher on days 4–7 of pregnancy. Although the recovery of body weight gain was observed after the administration of DBTCl on days 0–3 of pregnancy, recovery by the end of the study was not found in females given DBTCl at 7.6 mg/kg bw/day and higher after the administration on days 4–7 of pregnancy. Following the administration on days 4–7 of pregnancy, a significantly lower adjusted weight gain was also noted in females given DBTCl at 15.2 mg/kg/day and higher. These findings indicate that more severe adverse effects on body weight gain were induced by DBTCl on days 4–7 of pregnancy than that on days 0–3 of pregnancy. More severe effects of DBTCl on body weight gain following the administration on days 4–7 may be attributable to the significant decrease in the number of live fetuses.

The earlier administration period, days 0–3 of pregnancy, corresponds to the period before implantation, and the later administration period, days 4–7 of pregnancy, corresponds to the period when implantation is in progress and the period shortly after implantation in mice (Rugh, 1968). We expected that DBTCl insult on days 0–3 of pregnancy might result in preimplantation loss of embryos; i.e., the absence or decrease of implantation sites, and DBTCl insult on days 4–7 of pregnancy might result in postimplantation loss of embryos; i.e.,

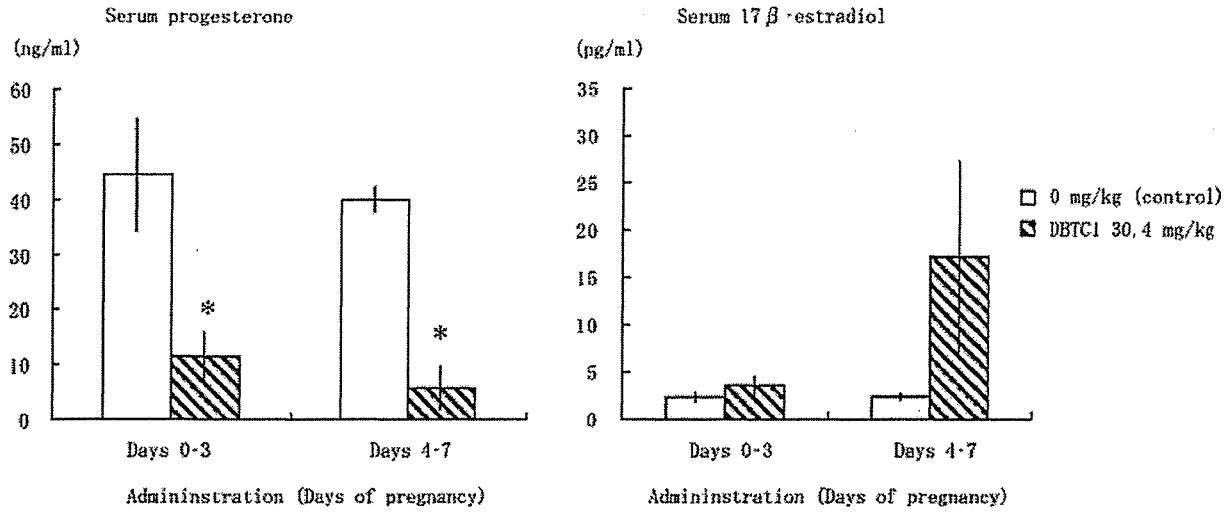

Fig. 1. Serum progesterone and 17β-estradiol levels in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Blood samples were collected on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± SEM of seven or eight mice. *Significantly different from the control group, $P < 0.05$.

the resorption of implantation sites. The most striking adverse effects of DBTCl on reproduction and development were a decrease in pregnancy rate, complete implantation failure, when DBTCl was given to mice on days 0–3 of pregnancy. The findings of an increased incidence of preimplantation embryonic loss in successfully mated females, and an increased incidence of postimplantation embryonic loss and low fetal weight in pregnant females survived until scheduled sacrifice after the administration of DBTCl on days 0–3 of pregnancy may suggest that DBTCl adversely affects preimplantation embryos and also the later survival and growth of embryos/fetuses when administered during the preimplantation period. On the other hand, the predominant adverse effects of DBTCl on reproduction and development were postimplantation loss, complete litter loss, when DBTCl was given to mice on days 4–7 of pregnancy. The findings of an increase in the incidence of postimplantation embryonic loss and a decrease in the fetal weight after administration of DBTCl on days 4–7 of pregnancy may suggest that DBTCl has effects on the later survival and growth of embryos/fetuses when administered during the peri-implantation period. Considered collectively, these findings indicate that the manifestation of adverse effects of DBTCl on reproduction and development varies with the stages of pregnancy at the time of maternal exposure.

The corpora lutea are essential up to the end of pregnancy in mice (Deansely, 1966). The embryo transport process in mice is triggered by progesterone and requires progesterone activity for its maintenance (Kendle and Lee, 1980). In mice, 24 h of progesterone priming is not only adequate for implantation, but this priming has a long-term effect on implantation (Huet-Hudson and Dey, 1990). In our previous studies in rats, increases in the incidences of early embryonic loss were observed after the administration of DBTCl during early pregnancy (Ema and Harazono, 2000ab). The suppression of uterine decidualization and reduced levels of serum progesterone were found in female rats given DBTCl on days 0–3 or days 4–7 of pseudopregnancy (Harazono and Ema, 2003), and lowered reproductive parameters in female rats given DBTCl were recovered by the administration of progesterone (Ema et al., 2003). Based on these findings, we hypothesized that the decline in serum progesterone levels in pregnant animals was a primary mechanism for the implantation failure due to DBTCl in rats. In the present study in mice, a decline in serum progesterone levels was detected after the administration of DBTCl during early pregnancy. These findings are in good agreement with previous findings that DBTCl induced early embryonic loss and decreased serum progesterone levels in pregnant rats. There is a similarity in the effects of DBTCl on progesterone levels in early pregnancy in rats and mice, and these suggest that the decline in the serum progesterone levels is also the factor responsible for the DBTCl-induced pregnancy failure in mice. Early pregnancy failure was also caused by systemic activation of the CD-40 immune costimulatory pathway in mice (Erlebacher et al., 2004). They noted that pregnancy failure resulted from impaired progesterone synthesis by the corpus luteum of the ovary, an endocrine defect in turn associated with ovarian resistance to the gonadotropic effects of prolactin and that pregnancy failure also required the proinflammatory cytokine TNF-a and correlated with the luteal induction of the prolactin receptor signaling inhibitors suppressor of cytokine signaling 1

(Socs1) and Socs3. Our results of the present study may support their argument. To further evaluate the adverse effects of DBTCl during early pregnancy, determination of the gene expression profile in the uterus of mice and rats is currently in progress.

In conclusion, DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice, and the present data suggest that the decline in progesterone is the responsible factor for the early pregnancy failure in mice.

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Evaluation of Developmental Toxicity of Ultraviolet Absorber 2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-Chlorobenzotriazole in Rats

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2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as a UV absorber. In this study, the developmental toxicity of DBHCB was evaluated in rats. Pregnant rats were given DBHCB at 0, 62.5, 250, or 1000 mg kg^{-1} day^{-1} by gavage on days 5–19 of pregnancy. No deaths were observed in the pregnant rats of any group. No effect of DBHCB on the general conditions, body weight gain, or feed consumption was observed in the pregnant rats. There were no changes in the ovarian weight, gravid uterine weight, or necropsy findings in the maternal rats of the DBHCB-treated groups. No significant effects of DBHCB were found in the number of corpora lutea, implantations, live fetuses, resorptions or dead fetuses, incidence of pre- or postimplantation embryonic loss, viability of fetuses, fetal weight, or sex ratio of live fetuses. No significant difference in the incidence of fetuses with malformations or variations or degree of ossification was detected between the DBHCB-treated and control groups.

Keywords Benzotriazole, Developmental toxicity, Rat, UV absorber.

INTRODUCTION

2-(3',5'-Di-*tert*-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole (CAS no. 3864 99–1; DBHCB) is slightly yellowish powder, stable under ordinary conditions, and insoluble in water. Its melting point is 154–158°C, and its specific gravity

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is 1.26. This chemical provides effective light stabilization and prevents the yellowing and degradation of polymers such as polypropylene, high-density polyethylene, unsaturated polyester, styrene-based thermoplastics elastomer, polyamide and impact polystyrene and is used as a UV absorber (Chemical Land21, 2005). The finished polymers—which contain UV absorbers at levels not to exceed 0.5% by weight of polyethylene phthalate polymers, complying with 21 CFR 177.1630 (FDA, 2005a)—may be used in contact with some food types and used under certain conditions as described in 21 CFR 176.170 (FDA, 2000; 2005b). UV absorbers are used in food packages as plastic additives, their function being mainly to prevent polymer degradation and/or a change in the quality of the packed food due to UV rays.

It has caused some anxiety that humans have been exposed to these chemicals in occupational surroundings, from environmental contamination and from contamination in food migrated from packages. The possibility of these chemicals entering the biological system has aroused great concern about their toxic potential. Important information can be gained by studying the biological effects produced by environmental chemicals in laboratory animals, in order to investigate their possible influences on human health.

Recently, DBHCB was assessed for its estrogenic activity, using a recombinant yeast assay (Miller et al., 2001) and the yeast two-hybrid assay (Kawamura et al., 2003); it was reported that DBHCB was not estrogenic. Some information on toxicity is available (Everlight Chemical Industrial Corporation, 2002). The oral LD_{50} for DBHCB was greater than 5000 mg/kg in rats. DBHCB caused minimal irritation to the skin and slight irritation to the eyes in rabbits. A 90-day feeding study of DBHCB in rats, at 22–800 mg/kg, resulted in dose-dependent increases in liver weights and signs of liver toxicity. No effects were found at 3.7 mg/kg. However, no detailed information is available for the toxicity studies.

Although testing for reproductive and developmental toxicity has become an important part of the overall toxicology profile for chemicals, no information has yet been presented on the reproductive and developmental toxicity of DBHCB. Therefore, the current study was conducted to evaluate the developmental toxicity of DBHCB given orally to rats during pregnancy.

MATERIALS AND METHODS

This study was performed in compliance with the OECD Guideline 414 Prenatal Developmental Toxicity Study (OECD, 2001) in 2004 at the Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan).

Animals

International Genetic Standard [Crj: CD (SD) IGS] rats were used throughout this study. This strain was chosen because it is most commonly

used in reproductive and developmental toxicity studies, and historical control data are available. Males at 11 weeks of age and females at 10 weeks of age were purchased from Hino Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for 1 week prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared with a basal diet (CE-2; Clea Co., Ltd., Tokyo, Japan), water was provided *ad libitum*, and the animals were maintained in an air-conditioned room at 21.6–22.2°C, with a relative humidity of 45–58%, a 12-h light/dark cycle, and ventilation with 15 air changes/hour. Virgin female rats were mated overnight with male rats. The day when the sperm and/or vaginal plug was found to be day 0 of pregnancy. The copulated females, weighing 245–314 g, 11 weeks old, were distributed on a random basis into 4 groups of 20 rats each and housed individually. This experiment was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the committee of SNBL.

Chemicals and Dosing

DBHCB was obtained from Musashino Geigy Co., Ltd. (Kitaibaraki, Japan). The DBHCB (lot no. 05004IX3) used in this study was 99.9% pure based on HPLC analysis, and it was kept in a dark place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before the study. Rats were treated once daily by gastric intubation with DBHCB at a dosage of 0 (control), 62.5, 250, or 1000 mg/kg on day 5 through day 19 of pregnancy. The dosage levels were determined based on the results of our dose-finding study in which a significantly increased liver weight was caused in males at 250 mg kg^{-1} day^{-1} and higher, but not in females even at 1000 mg kg^{-1} day^{-1}, after administration of DBHCB for 14 days in rats. DBHCB was suspended in 5% gum arabic solution. The volume of each dose was adjusted to 10 mL/kg body weight based on the latest body weight. The control rats were given only 5% gum arabic solution. The stability of the formulations in a dark and cool place under airtight conditions had been confirmed for up to 14 days. During use, the formulations were maintained under such conditions for no more than 7 days and were 97.3% to 100.1% of the target concentration.

Observations

All females were observed daily during the preadministration period and twice a day (before administration and 1 to 2 h after administration) during the administration period for clinical signs of toxicity. Maternal body weight was recorded on days 0, 5, 8, 11, 14, 17, 19, and 20 of pregnancy. Feed consumption was recorded on days 0–1, 5–6, 8–9, 11–12, 14–15, 17–18, and 19–20

of pregnancy. The pregnant rats were euthanized by exsanguination under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the uterus and ovaries were removed from the maternal body and weighed. The numbers of corpora lutea, implantation sites, and live and dead fetuses and resorptions were counted. The live fetuses were removed from the uterus and sexed, weighed, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected, fixed in alcohol, stained with alizarin red S (Dawson, 1926), and examined for skeletal anomalies. The remaining live fetuses in each litter were fixed in Bouin's solution. Their heads were subjected to free-hand razor-blade sectioning (Wilson, 1973), and the thoracic areas were subjected to microdissecting (Nishimura, 1974) to reveal internal abnormalities.

Data Analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. The initial body weight, body weight gain, and feed consumption of the pregnant rats, numbers of corpora lutea, implantations and live fetuses per litter, and fetal weight were analyzed with Bartlett's test (Snedecor and Cochran, 1974) for homogeneity of variance at the 5% level of significance. When the variance was homogeneous, Dunnett's test (Dunnett, 1996) was performed to compare the mean value in the control group with that in each DBHCB group. When the variance was heterogeneous, a Dunnett-type test (Miller, 1987) was performed to compare the mean value in the control group with that in each DBHCB group after rank conversion. The Dunnett-type test was used for the incidences of pre- and postimplantation embryonic loss and fetal anomalies and sex ratio of fetuses to compare the mean rank of groups treated with DBHCB and that of the control group. The incidence of dams with anomalous fetuses was analyzed with Fisher's exact test.

RESULTS

Table 1 shows the maternal findings in rats given DBHCB on days 5–19 of pregnancy. No deaths or clinical signs of toxicity were found in female rats of any group. There was no difference in the fertility rate between the control and DBHCB-treated groups. No effects of DBHCB on body weight gains on days 0–5, 5–14, 14–19, and 19–20 of pregnancy were observed. During the whole period of pregnancy, no effects of DBHCB were also detected in body weight gain. There was no difference in feed consumption during pregnancy between the control and DBHCB-treated groups. No effects of DBHCB on weights of the gravid uterus and ovaries were detected.

Table 1: Maternal findings in rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)			
	0 (control)	**62.5**	**250**	**1000**
No. of rats	20	20	20	20
No. of pregnant rats	17	18	17	18
No. of dead rats	0	0	0	0
Initial body weight	285 ± 11	280 ± 12	285 ± 18	288 ± 11
Body weight gain during pregnancy (g)[a]				
Days 0–5	30 ± 8	33 ± 5	31 ± 6	30 ± 6
Days 5–14	47 ± 7	44 ± 7	49 ± 5	43 ± 9
Days 14–19	71 ± 9	65 ± 10	67 ± 10	63 ± 12
Days 19–20	16 ± 6	17 ± 4	20 ± 5	18 ± 5
Days 0–20	163 ± 17	159 ± 19	167 ± 14	154 ± 20
Adjusted weight gain[b]	88 ± 9	88 ± 10	91 ± 10	82 ± 18
Feed consumption during pregnancy (g/day)[a]				
Days 0–1	24 ± 3	23 ± 3	23 ± 3	24 ± 4
Days 5–6	27 ± 3	27 ± 3	27 ± 3	27 ± 3
Days 8–9	28 ± 4	28 ± 3	28 ± 3	28 ± 2
Days 11–12	29 ± 4	29 ± 3	28 ± 2	29 ± 3
Days 14–15	28 ± 4	28 ± 3	28 ± 3	28 ± 3
Days 17–18	32 ± 4	30 ± 4	31 ± 3	31 ± 4
Days 19–20	29 ± 4	29 ± 3	31 ± 4	30 ± 3
Weight of gravid uterus (g)[a]	88 ± 9	88 ± 10	91 ± 10	82 ± 18
Weight of ovaries (mg)[a]	149 ± 21	137 ± 14	149 ± 19	139 ± 14

[a]Values are given as the mean ± SD.
[b]Adjusted weight gain refers to maternal weight gain excluding the gravid uterus.

The reproductive findings in rats given DBHCB on days 5–19 of pregnancy are presented in Table 2. No totally resorbed litters were found in any group. No effects of DBHCB were observed on the number of corpora lutea or implantations, incidence of pre- or postimplantation loss, or the number of live fetuses or the sex ratio of live fetuses. There was no difference in the body weight of male and female fetuses between the control and DBHCB-treated groups. No abnormal findings were noted in the placentae of any group.

Morphological findings in the live fetuses of rats given DBHCB on days 5–19 of pregnancy are shown in Table 3. No fetuses with external malformations were observed in any group. Skeletal examination revealed no fetuses with skeletal malformations in any group. Fetuses with skeletal variations were observed in all groups including the control group. The incidence of fetuses with individual skeletal variations was not increased after the administration of DBHCB. The total number of fetuses with skeletal variations was also not increased in the DBHCB-treated groups. The degree of ossification, as evidenced by the numbers of sacral and caudal vertebrae and sternebrae in the DBHCB-treated groups, was not different from that in the control group. No fetuses with internal malformations were detected in any group. The fetuses with internal variations, such as thymic remnants in the neck, dilated renal