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A 28-Day Repeated Dose Toxicity Study of Ultraviolet Absorber 2-(2′-Hydroxy -3′,5′-di-*tert*-butylphenyl) benzotriazole in Rats

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To examine the possible repeated-dose toxicity of an ultraviolet absorber, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), CD(SD)IGS rats were administered HDBB by gavage at a dose of 0 (vehicle: corn oil), 0.5, 2.5, 12.5, or 62.5 mg kg^{-1} day^{-1} for 28 days. At the completion of the administration period, a decrease in red blood cells, hemoglobin, and hematocrit was noted only in males at 2.5 mg/kg and more. Blood biochemical changes were noted at 0.5 mg/kg and more in males and at 62.5 mg/kg in females. Histopathologic changes were observed principally in the liver (vacuolar degeneration and hypertrophy of hepatocytes, bile duct proliferation, etc.) and in the heart (degeneration and hypertrophy of myocardium and cell infiltration). These changes were noted at 0.5 mg/kg and more in males and at 12.5 mg/kg and more in females. At higher doses, hypertrophy of tubular epithelium in the kidneys and diffuse follicular cell hyperplasia in the thyroids in both sexes and increased severity of basophilic tubules in the kidneys and extramedullary hematopoiesis in the spleen in males were also detected. After the 14-day recovery period, these changes mostly recovered in females but not in males. Based on these findings, no observed adverse effect level (NOAEL) was concluded to be less than 0.5 mg kg^{-1} day^{-1} in male rats and 2.5 mg kg^{-1} day^{-1} in female rats.

Keywords Benzotriazole, Gender-related difference, Rat, Repeated dose toxicity, UV absorber

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INTRODUCTION

Plastic generally ages rapidly under the effects of light, oxygen, and heat, leading to loss of strength, reduced flexibility and electric properties, discoloration, scratching, and loss of gloss (Commerce Online, 2006; Tenkazai.com, 2006). In particular, ultraviolet (UV), possessing considerable energy (e.g., approximately 70 kcal/mol at 400 nm and 110 kcal/mol at 250 nm), directly breaks polymer bonds and promotes oxidative degradation in the presence of oxygen; therefore, UV absorbers are added to plastics to improve their long-term weather resistance and stability.

Benzotriazole UV absorbers, which have a phenolic group attached to the benzotriazole structure, are known to have the most excellent absorption capacity with a full spectrum of UV absorption (Tenkazai.com, 2006), and are therefore used in a variety of polymers. In 1999, the Phenolic Benzotriazole Association voluntarily agreed to participate in the U.S. High Production Volume Chemical Challenge Program (U.S. EPA, 2001). The existing data on four benzotriazole UV absorbers (2-(2′-hydroxy-5′-methylphenyl)benzotriazole, 2-(2′-hydroxy-5′-octylphenyl)benzotriazole, 2-(2′-hydroxy-3′,5′-di-*tert*-amylphenyl)benzotriazole, and 2-(2H-benzotriazole-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol), reviewed in this program, showed low acute mammalian toxicity, moderate toxicity with repeated exposure (effect typically in the liver and kidney), and a lack of genotoxicity in this category of chemicals.

2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (CAS no. 3846–71–7; HDBB) is a benzotriazole UV absorber added at ~0.02–2% mainly to unsaturated polyester resin, polycarbonate, vinyl chloride resin, polyacrylic acid ester, polyacetal, polyolefin, polymethacrylic acid ester, and polyamide (METI, 2006). From these resins, plastic resin products such as building materials and automobile components are manufactured. In addition, HDBB is also used in printing or sensitive materials and coating compounds, all intended for UV absorption. Although 257.5 tons were produced in Japan from April 2002 to March 2003, only limited toxicity information as a short abstract written in Japanese, which was distributed to the Committee on Safety of Chemical Substances in Chemical Substances Council of Japan, was available (METI, 2006). HDBB was selected as an object substance in an existing chemical testing program by the Japanese Government (MHLW, 2003). In this program, a 28-day repeated-dose toxicity study of HDBB was performed using rats to obtain information on its toxicity. We report the details here.

MATERIALS AND METHODS

This study was performed in compliance with the Test Guideline of the Japanese Chemical Control Act (law concerning examination and regulation of manufacture, etc., of chemical substances), "Twenty-eight-day Repeated Dose

Toxicity Test in Mammalian Species" (EA, MHW and MITI, 1986), and in accordance with the principles for Good Laboratory Practice (OECD, 1998; EA, MHW and MITI, 2000) at the Biosafety Research Center, Foods, Drugs and Pesticides (An-pyo Center, Iwata, Japan).

Chemicals

HDBB was obtained from Shipro Kasei Kaisha, Ltd. (Osaka, Japan). The HDBB (lot no. S4-034-1) used in this study was 100 wt% pure, and it was kept at room temperature. Test solutions were prepared as suspension in corn oil once a week and kept cool until dosing because stability for 7 days was confirmed under these conditions. The concentration was adjusted in such a way that the volume of each dose is constantly 5 mL/kg based on the latest body weight. The test solutions were confirmed to be 94.2% to 104.3% of the target concentration by analysis using high-performance liquid chromatography. All other reagents used in this study were specific purity grade.

Animals

Crj;CD (SD) IGS rats (SPF, 4 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animals were maintained in an air-conditioned room at 21.4–25.9°C, with a relative humidity of 51–75%, a 12-h light/dark cycle, and ventilation with 20 air changes per hour. They were housed individually in stainless steel wire mesh cages with anterior surfaces of aluminum. A basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. Male and female rats were assigned to each dose group by stratified random sampling based on body weight. The initial numbers of rats were 10/sex in control and the highest dose group, and 5/sex in other dose groups. After 8-day acclimation, they were subjected to treatment at 5 weeks of age. This experiment was approved by the Institutional Animal Care and Use Committee of An-pyo Center and performed in accordance with the ethics criteria contained in the bylaws of the committee of An-pyo Center.

Experimental Design

The dosage levels were determined based on the findings in a 14-day dose-finding study, in which an increase in absolute and relative liver weight was observed at all doses of 100, 300, and 1000 mg kg^{-1} day^{-1}. Rats were given HDBB once daily at 0 (vehicle control), 0.5, 2.5, 12.5, or 62.5 mg kg^{-1} day^{-1} by gavage for 28 days. The day after the last dosing, five males and five females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The remaining five rats/sex at 0 and 62.5 mg/kg were kept without treatment for 14 days as a recovery period and then fully examined.

All animals were observed before and 1 h and 5 h after dosing for clinical signs of toxicity. During the recovery period, observation was made twice a day (morning and afternoon). Body weight was recorded on days 0, 7, 14, 21, and 27 of the dosing period and days 0, 7, and 13 of the recovery period. Food consumption was measured on days 7, 14, 21, and 27 of the dosing period and days 7 and 13 of the recovery period. On day 25 of the dosing period and day 11 of the recovery period, urine was collected for 3 h and analyzed for dipstick parameters, such as occult blood, pH, protein, glucose, ketone bodies, bilirubin, and urobilinogen. In addition, a 24-h urine sample was also collected for color, sediment, osmotic pressure, and volume of the urine.

Prior to necropsy at the end of dosing and recovery periods, blood was collected from the abdominal aorta under deep ether anesthesia after overnight starvation. One portion of the blood was treated with EDTA-2K and examined for hematologic parameters such as red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count, platelet count, reticulocyte count, and differential leukocyte count. Another blood sample was treated with 3.13% sodium citrate, and blood clotting parameters such as prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were examined. Serum from the remaining portions of blood was analyzed for blood biochemistry [total protein, albumin, albumin-globulin (A/G) ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase, calcium, inorganic phosphorus, sodium, potassium, chlorine]. After the collection of blood, all animals were sacrificed by exsanguination, and the surface and cavity of the body and the organs and tissues of the entire body were macroscopically observed. The brain, pituitary, thymus, thyroids (including parathyroids), heart, liver, spleen, kidneys, adrenals, testes, epididymides, and ovaries were then removed and weighed (after formalin fixation of the pituitary and thyroids). The trachea, lungs (including bronchus), pancreas, lymph nodes (mesenteric and mandibular), stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, spinal cord (cervical, pectoral, and lumbar part), sciatic nerve, seminal vesicles, prostates, uterus, vagina, bone marrow (femur), skeletal muscle (femur) as well as the above organs were fixed in 10% neutral-buffered formalin phosphate (after formalin acetate fixation for testes and epididymides). Histopathologic examination was conducted for all these organs of the control and the highest dose groups. In addition, the liver, heart, kidneys, spleen, and thyroids of the other groups were examined, as test-substance-related changes were found in the highest group. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin.

Data Analysis

Parametric data such as body weight, food consumption, urinalysis findings (urine volume and osmotic pressure), hematologic and biochemical findings, and organ weights were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett, 1964) was conducted for comparison between control and individual treatment groups. If not homogenous, the data were analyzed using Steel's multiple comparison test (Steel, 1959). For histopathologic findings, Fisher's exact test (Fisher, 1973) was performed. The 5% level of probability was used as the criterion for significance.

RESULTS

No death or clinical sings of toxicity were found in any groups. There were also no significant changes in body weight, but a significant increase in food consumption was noted on dosing days 14 and 21 in males and on dosing days 21 and 27 in females at 62.5 mg/kg. No dose-related changes were found in the findings of urinalysis.

At the end of the 28-day administration period, a significant decrease in red blood cell count, hematocrit and hemoglobin at 2.5 mg/kg and more, decrease in MCHC at 12.5 mg/kg and more, and increase in platelet count at 62.5 mg/kg were noted in males, but these changes were not found in females (Table 1). For clotting factors, a significant decrease in fibrinogen was noted at 2.5 mg/kg and more in males and at 62.5 mg/kg in females (Table 1) but no significant prolongation of PT or APTT.

Blood biochemical examination revealed significant increases in the A/G ratio at 0.5 mg/kg and more, and levels of glucose at 2.5 mg/kg and more, albumin, ALT, and ALP at 12.5 mg/kg and more, and BUN and AST at 62.5 mg/kg in males (Table 2). On the other hand, for females, a significant increase in the levels of glucose, A/G ratio, total cholesterol, triglyceride, and ALT was noted only at 62.5 mg/kg.

At necropsy, absolute liver weight was significantly increased at 2.5 mg/kg and more in males and at 12.5 mg/kg and more in females with a significant increase in the relative weight at all doses in males and at 12.5 mg/kg and more in females (Table 3). In the highest dose group, there was also a significant increase in absolute and relative kidney weight in males and in absolute heart weight in females. No test-substance-related significant change was detected in other organs. Macroscopically, enlargement of the liver was observed at all doses in males and at 12.5 mg/kg and more in females. In the liver, a white patch/zone was found at 2.5 mg/kg and more in males and at 62.5 mg/kg in females.

Table 1: Principal hematologic values in male and female rats given HDBB by gavage for 28 days.

	At the completion of the administration period					At the completion of the recovery period	
	0 mg kg^{-1} day^{-1}	0.5 mg kg^{-1} day^{-1}	2.5 mg kg^{-1} day^{-1}	12.5 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}	0 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}
Male							
No. of animals	5	5	5	5	5	5	5
Red blood cells ($10^6/mm^3$)	7.89 ± 0.18	7.65 ± 0.32	7.23 ± 0.33*	7.18 ± 0.27**	7.16 ± 0.46**	8.26 ± 0.16	7.65 ± 0.38*
Hemoglobin (g/dL)	15.2 ± 0.4	14.8 ± 0.5	13.9 ± 0.8**	13.6 ± 0.3**	13.2 ± 0.3**	15.3 ± 0.3	13.2 ± 0.9**
Hematocrit (%)	45.6 ± 1.8	44.6 ± 1.5	42.5 ± 2.4*	41.9 ± 1.2**	40.7 ± 0.9**	44.6 ± 1.0	40.1 ± 2.7**
MCV (μm^3)	57.8 ± 1.9	58.3 ± 1.5	58.7 ± 1.2	58.3 ± 1.7	57.0 ± 2.7	54.0 ± 1.6	52.5 ± 2.6
MCH (pg)	19.3 ± 0.7	19.4 ± 0.6	19.3 ± 0.4	19.0 ± 0.9	18.4 ± 0.9	18.5 ± 0.5	17.3 ± 0.9*
MCHC (%)	33.4 ± 0.7	33.2 ± 0.5	32.8 ± 0.2	32.5 ± 0.7*	32.3 ± 0.3*	34.2 ± 0.3	32.9 ± 0.6**
Reticulocyte (%)	2.8 ± 0.3	3.3 ± 0.4	3.2 ± 0.3	3.9 ± 0.5*	3.2 ± 1.0	2.5 ± 0.4	4.4 ± 0.2**
Platelet count ($10^3/mm^3$)	1202 ± 75	1265 ± 107	1280 ± 116	1572 ± 430	1639 ± 227*	1196 ± 145	1502 ± 134**
Fibrinogen (mg/dL)	249 ± 13	224 ± 8	189 ± 15**	198 ± 21**	193 ± 20**	240 ± 24	214 ± 13
Female							
No. of animals	5	5	5	5	5	5	5
Red blood cells ($10^6/mm^3$)	7.81 ± 0.38	7.62 ± 0.61	7.79 ± 0.22	7.46 ± 0.30	7.49 ± 0.30	7.80 ± 0.27	7.64 ± 0.38
Hemoglobin (g/dL)	15.1 ± 0.9	14.9 ± 1.3	15.2 ± 0.4	14.8 ± 0.7	14.1 ± 0.6	14.9 ± 0.5	14.2 ± 0.6
Hematocrit (%)	43.7 ± 1.7	43.5 ± 3.1	44.0 ± 1.3	43.1 ± 1.8	41.6 ± 1.6	42.2 ± 1.0	40.6 ± 1.6
MCV (μm^3)	56.0 ± 1.1	57.1 ± 1.6	56.4 ± 0.8	57.7 ± 1.4	55.6 ± 1.0	54.2 ± 0.8	53.2 ± 1.8
MCH (pg)	19.3 ± 0.4	19.6 ± 0.6	19.5 ± 0.4	19.8 ± 0.5	18.9 ± 0.4	19.1 ± 0.3	18.6 ± 0.6
MCHC (%)	34.5 ± 0.8	34.3 ± 0.8	34.5 ± 0.4	34.4 ± 0.3	34.0 ± 0.4	35.2 ± 0.3	35.1 ± 0.4
Reticulocyte (%)	2.1 ± 0.4	3.5 ± 1.7	2.6 ± 0.4	2.5 ± 0.2	2.4 ± 0.3	2.7 ± 0.4	2.6 ± 0.3
Platelet count ($10^3/mm^3$)	1295 ± 118	1360 ± 155	1367 ± 79	1368 ± 138	1350 ± 194	1166 ± 64	1410 ± 95**
Fibrinogen (mg/dL)	193 ± 11	222 ± 46	186 ± 9	184 ± 29	155 ± 10*	210 ± 7	241 ± 7**

Values are expressed as the mean ± SD.
*Significantly different from the control, $p \leq 0.05$.
**Significantly different from the control, $p \leq 0.01$.

Table 2: Principal blood biochemical values in male and female rats given HDBB by gavage for 28 days.

	At the completion of the administration period					At the completion of the recovery period	
	0 mg kg^{-1} day^{-1}	0.5 mg kg^{-1} day^{-1}	2.5 mg kg^{-1} day^{-1}	12.5 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}	0 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}
Male							
No. of animals	5	5	5	5	5	5	5
Total protein (g/dL)	5.84 ± 0.34	5.52 ± 0.10	5.55 ± 0.24	5.72 ± 0.22	5.86 ± 0.40	6.02 ± 0.19	5.95 ± 0.49
Albumin (g/dL)	3.78 ± 0.22	3.90 ± 0.17	4.06 ± 0.20	4.43 ± 0.18**	4.40 ± 0.41**	3.75 ± 0.1	4.22 ± 0.45*
A/G ratio	1.85 ± 0.18	2.43 ± 0.23*	2.75 ± 0.29**	3.47 ± 0.25**	3.05 ± 0.55**	1.66 ± 0.11	2.46 ± 0.34*
Glucose (mg/dL)	122 ± 13	132 ± 15	170 ± 18**	170 ± 10**	156 ± 16**	166 ± 13	182 ± 22
Total cholesterol (mg/dL)	59 ± 11	46 ± 9	45 ± 4	49 ± 13	52 ± 20	62 ± 13	55 ± 19
Triglyceride (mg/dL)	25.5 ± 8.4	24.3 ± 4.5	34.5 ± 7.1	44.8 ± 20.9	45.8 ± 12.5	68.0 ± 52.0	47.5 ± 26.6
BUN (mg/dL)	13.0 ± 2.5	12.9 ± 0.5	15.5 ± 1.7	15.8 ± 1.3	17.2 ± 2.4**	14.5 ± 2.4	19.0 ± 1.9*
AST (U/L)	72 ± 7	71 ± 11	65 ± 5	83 ± 22	115 ± 16*	61 ± 7	68 ± 22
ALT (U/L)	30 ± 5	28 ± 4	32 ± 3	42 ± 5*	48 ± 10**	25 ± 5	49 ± 29**
ALP (U/L)	757 ± 175	992 ± 220	1089 ± 168	1569 ± 427**	1462 ± 250**	622 ± 123	906 ± 169*
Female							
No. of animals	5	5	5	5	5	5	5
Total protein (g/dL)	5.68 ± 0.14	5.61 ± 0.18	5.53 ± 0.19	5.93 ± 0.33	5.85 ± 0.19	5.91 ± 0.29	6.50 ± 0.30*
Albumin (g/dL)	3. 81 ± 0.23	3.67 ± 0.43	3.72 ± 0.12	4.12 ± 0.14	4.21 ± 0.18	3.85 ± 0.32	4.27 ± 0.10*
A/G ratio	2.04 ± 0.26	1.95 ± 0.44	2.09 ± 0.27	2.30 ± 0.25	2.59 ± 0.29*	1.89 ± 0.25	1.93 ± 0.18
Glucose (mg/dL)	110 ± 15	120 ± 20	114 ± 16	127 ± 22	151 ± 8**	117 ± 8	149 ± 16**
Total cholesterol (mg/dL)	49 ± 10	59 ± 5	50 ± 7	54 ± 6	84 ± 16**	63 ± 6	91 ± 14**
Triglyceride (mg/dL)	12.3 ± 5.6	12.1 ± 2.6	8.8 ± 3.7	12.2 ± 1.1	31.9 ± 4.8**	18.8 ± 7.6	37.7 ± 18.8
BUN (mg/dL)	16.1 ± 4.3	15.5 ± 1.5	16.6 ± 3.8	15.8 ± 2.4	16.9 ± 1.3	16.6 ± 1.2	16.8 ± 0.8
AST (U/L)	68 ± 5	69 ± 11	66 ± 7	68 ± 9	76 ± 12	66 ± 13	65 ± 19
ALT (U/L)	21 ± 2	22 ± 4	23 ± 3	27 ± 4	33 ± 6**	25 ± 4	36 ± 21
ALP (U/L)	490 ± 110	409 ± 86	414 ± 85	433 ± 83	633 ± 199	381 ± 138	247 ± 63

Values are expressed as the mean ± SD.
*Significantly different from the control, $p \leq 0.05$.
**Significantly different from the control, $p \leq 0.01$.

Table 3: Principal organ weights of male and female rats given HDBB by gavage for 28 days.

	At the completion of the administration period					At the completion of the recovery period	
	0 mg kg^{-1} day^{-1}	0.5 mg kg^{-1} day^{-1}	2.5 mg kg^{-1} day^{-1}	12.5 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}	0 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}
Male							
No. of animals	5	5	5	5	5	5	5
Brain (g)	2.02 ± 0.08	2.03 ± 0.07	2.12 ± 0.06	2.08 ± 0.05	2.06 ± 0.09	2.10 ± 0.10	2.07 ± 0.10
	(0.624 ± 0.009)[a]	(0.622 ± 0.038)	(0.633 ± 0.062)	(0.628 ± 0.044)	(0.630 ± 0.046)	(0.527 ± 0.046)	(0.580 ± 0.034)
Heart (g)	1.09 ± 0.09	1.10 ± 0.11	1.17 ± 0.14	1.18 ± 0.07	1.23 ± 0.19	1.20 ± 0.10	1.28 ± 0.16
	(0.337 ± 0.026)	(0.336 ± 0.028)	(0.346 ± 0.011)	(0.355 ± 0.017)	(0.374 ± 0.028)	(0.298 ± 0.008)	(0.356 ± 0.016**)
Liver (g)	9.40 ± 0.58	11.65 ± 1.90	17.11 ± 3.46*	21.64 ± 2.73*	24.47 ± 5.06*	11.8 ± 1.64	20.61 ± 3.36**
	(2.908 ± 0.139)	(3.533 ± 0.296*)	(5.045 ± 0.506*)	(6.507 ± 0.536*)	(7.413 ± 1.283*)	(2.930 ± 0.133)	(5.746 ± 0.527**)
Kidneys (g)	2.43 ± 0.22	2.54 ± 0.17	2.74 ± 0.29	2.88 ± 0.40	3.04 ± 0.45*	2.83 ± 0.23	2.91 ± 0.40
	(0.753 ± 0.075)	(0.775 ± 0.046)	(0.814 ± 0.053)	(0.865 ± 0.080)	(0.927 ± 0.119**)	(0.706 ± 0.046)	(0.814 ± 0.066*)
Testes (g)	2.90 ± 0.16	2.84 ± 0.12	2.88 ± 0.15	2.91 ± 0.15	2.92 ± 0.14	3.13 ± 0.11	3.07 ± 0.18
	(0.901 ± 0.080)	(0.871 ± 0.084)	(0.865 ± 0.121)	(0.879 ± 0.046)	(0.891 ± 0.068)	(0.787 ± 0.099)	(0.861 ± 0.043)
Female							
No. of animals	5	5	5	5	5	5	5
Brain (g)	1.94 ± 0.10	1.92 ± 0.08	1.95 ± 0.07	1.90 ± 0.12	1.90 ± 0.03	1.99 ± 0.02	1.94 ± 0.05
	(0.931 ± 0.053)	(0.884 ± 0.012)	(0.901 ± 0.052)	(0.857 ± 0.046)	(0.841 ± 0.058*)	(0.838 ± 0.086)	(0.802 ± 0.084)
Heart (g)	0.75 ± 0.07	0.77 ± 0.03	0.75 ± 0.02	0.78 ± 0.05	0.84 ± 0.06*	0.79 ± 0.04	0.87 ± 0.06
	(0.357 ± 0.019)	(0.356 ± 0.008)	(0.348 ± 0.007)	(0.351 ± 0.009)	(0.371 ± 0.024)	(0.333 ± 0.022)	(0.357 ± 0.028)
Liver (g)	6.39 ± 0.87	6.84 ± 0.63	6.73 ± 0.26	8.67 ± 1.16**	12.43 ± 0.89**	6.80 ± 0.86	8.85 ± 0.99**
	(3.053 ± 0.178)	(3.146 ± 0.197)	(3.112 ± 0.107)	(3.885 ± 0.324**)	(5.497 ± 0.172**)	(2.836 ± 0.076)	(3.626 ± 0.117**)
Kidneys (g)	1.70 ± 0.14	1.61 ± 0.08	1.71 ± 0.09	1.72 ± 0.11	1.87 ± 0.19	1.77 ± 0.18	1.86 ± 0.13
	(0.816 ± 0.057)	(0.742 ± 0.033*)	(0.789 ± 0.029)	(0.776 ± 0.040)	(0.827 ± 0.042)	(0.744 ± 0.075)	(0.766 ± 0.070)
Ovaries (mg)	87 ± 22	96 ± 18	82 ± 11	97 ± 9	89 ± 18	88 ± 12	101 ± 11
	(0.041 ± 0.007)	(0.044 ± 0.008)	(0.038 ± 0.005)	(0.044 ± 0.005)	(0.039 ± 0.008)	(0.037 ± 0.004)	(0.041 ± 0.003)

Values are expressed as the mean ± SD.
[a] Relative organ weight (organ weight per body weight) (%).
*Significantly different from the control, $p \leq 0.05$.
**Significantly different from the control, $p \leq 0.01$.

On histopathology, test-substance-related changes were observed in the liver, heart, kidneys, thyroids, and spleen as shown in Table 4. In the liver, hypertrophy of hepatocytes in males at 0.5 mg/kg and more and in females at 12.5 and 62.5 mg/kg; bile duct proliferation and decreased incidence of hepatocellular fatty change in males at 0.5 mg/kg and more and in females at 62.5 mg/kg; vacuolar degeneration of hepatocytes in males at 2.5 mg/kg and more and in females at 62.5 mg/kg; focal necrosis in males at 2.5 mg/kg and more; increased mitosis of hepatocytes in males at 62.5 mg/kg and in females at 12.5 and 62.5 mg/kg; and hepatocellular pigmentation and/or cytoplasmic inclusion bodies in males at 62.5 mg/kg were observed. In the heart, cell infiltration at 2.5 mg/kg and more in males, and degeneration and/or hypertrophy of the myocardium at 12.5 and 62.5 mg/kg in both sexes were noted. Furthermore, hypertrophy of the tubular epithelium was observed in the kidneys of males at 12.5 and 62.5 mg/kg and of females at 62.5 mg/kg, and increased severity of basophilic tubules was found in males at 62.5 mg/kg. In the thyroids and spleen, diffuse follicular cell hyperplasia at 62.5 mg/kg in both sexes and extramedullary hematopoiesis at 2.5 mg/kg and more in males, respectively, were detected.

At the end of the recovery period, a significant decrease in red blood cell count, hematocrit, hemoglobin and MCHC, and increase in platelet count were still observed in males, and a significant decrease in MCH and increase in reticulocyte in males and increase in platelet count in females were additionally found (Table 1). A significant increase in serum levels of albumin, A/G ratio, BUN, ALT, and ALP in males, and in total protein, albumin, glucose and total cholesterol in females was also noted (Table 2). At necropsy, grossly enlarged liver was still observed, and the absolute and relative weight was significantly increased in both sexes (Table 3). In males, the liver was brown, and some were accompanied with a red or white patch/zone. A significant increase in the relative weight of the heart and kidneys was also noted in males (Table 3). Histopathologically, except for increased mitosis of hepatocytes, hepatic changes were observed with similar incidence as observed at the end of the administration period in males (Table 4). Degeneration of the myocardium and cell infiltration in the heart, diffuse follicular cell hyperplasia in the thyroid, and extramedullary hematopoiesis in the spleen were also detected in males. In females, hypertrophy of hepatocytes was found, but other histopathologic changes observed at the end of the administration period were not detected. In the liver, focal necrosis and hepatocellular pigmentation were also found in females.

DISCUSSION

The current study was conducted to obtain initial information on the possible repeated-dose toxicity of HDBB in rats. The dosage of HDBB used in this

Table 4: Histopathologic findings in the principal organs of male and female rats given HDBB by gavage for 28 days.

		At the completion of the administration period					At the completion of the recovery period	
	Grade	0 mg kg^{-1} day^{-1}	0.5 mg kg^{-1} day^{-1}	2.5 mg kg^{-1} day^{-1}	12.5 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}	0 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}
Male								
No. of animals		5	5	5	5	5	5	5
Liver								
Hypertrophy of hepatocytes	+	0	3	5**	5**	5**	0	5**
Fatty change of hepatocytes	+	5	0**	0**	0**	0**	5	0**
Bile duct proliferation	+	0	1	1	4*	4*	0	4*
Vacuolar degeneration of hepatocytes	+	0	0	5**	5**	5**	0	4*
Focal necrosis	+	0	0	1	2	4*	0	3
Increased mitosis of hepatocytes	+	0	0	0	0	4*	0	0
Pigment deposit of hepatocytes	+	0	0	0	0	1	0	2
Cytoplasmic inclusion bodies	+	0	0	0	0	1	0	1
Heart								
Cell infiltration	+	0	1	5**	4*	4*	1	3
Degeneration of myocardium	+	0	0	0	5**	5**	0	4*
Hypertrophy of myocardium	+	0	0	0	3	4*	0	0
Kidney								
Hypertrophy of tubular epithelium	+	0	0	0	2	5**	0	0
Basophilic tubules	+	2	3	4	3	3	4	4
	++	0	0	0	0	2	0	0
Thyroid								
Diffuse follicular cell hyperplasia	+	0	0	0	0	2	0	3

Spleen								
Extramedullary hematopoiesis	+	0	0	3	2	2	0	3
Female								
No. of animals		5	5	5	5	5	5	5
Liver								
Hypertrophy of hepatocytes	+	0	0	0	5**	5**	0	3
Fatty change of hepatocytes	+	5	5	5	3	0**	5	4
Bile duct proliferation	+	0	0	0	0	1	0	0
Vacuolar degeneration of hepatocytes	+	0	0	0	0	2	0	0
Focal necrosis	+	0	0	0	0	0	0	2
Increased mitosis of hepatocytes	+	0	0	0	1	2	0	0
Pigment deposit of hepatocytes	+	0	0	0	0	0	0	1
Heart								
Cell infiltration	+	0	1	0	0	1	0	0
Degeneration of myocardium	+	0	0	0	3	5**	0	0
Hypertrophy of myocardium	+	0	0	0	1	3	0	0
Kidney								
Hypertrophy of tubular epithelium	+	0	0	0	0	2	0	0
Basophilic tubules	+	1	2	2	0	3	4	4
Thyroid								
Diffuse follicular cell hyperplasia	+	0	0	0	0	2	0	0
Spleen								
Extramedullary hematopoiesis	+	0	1	0	0	0	0	0

Values represent the number of animals with findings.
+: slight; ++: moderate.
*Significantly different from the control, $p \leq 0.05$.
**Significantly different from the control, $p \leq 0.01$.

study was sufficiently high to be expected to induce toxicity in the liver. As expected, histopathologic changes including vacuolar degeneration and hypertrophy of hepatocytes were observed in the liver. These findings showed that one of the toxicologically main targets of HDBB was the liver. Increased food consumption without body weight changes, increased blood glucose, total cholesterol and triglyceride, and decreased incidence of fatty changes of hepatocytes were noted after HDBB administration for 28 days. These changes indicate metabolic derangement and suggest possible adverse effects of HDBB in metabolic homeostasis. The current study showed that the heart was another toxicologic target organ for HDBB. Although degeneration and hypertrophy of the myocardium and cell infiltration were observed after HDBB administration, cardiac function was not evaluated in the current study. Further studies are required to clarify the adverse effects of HDBB on cardiac function, because functional parameters are considered to be more susceptible than histopathologic changes in the heart (Glaister, 1992). In our study, HDBB also caused anemic changes (decreased red blood cell count, hematocrit, hemoglobin and MCHC, and extramedullary hematopoiesis), and adverse effects on the kidneys (hypertrophy of tubular epithelium and increased severity of basophilic tubules with increased BUN) and the thyroids (diffuse follicular cell hyperplasia) at higher doses. Adverse effects on the liver and kidneys, and anemia, but not adverse effects on the heart and thyroid, were reported in the 90-day repeated feeding study on the structural analogue, 2-(2′-hydroxy-3′,5′-di-*tert*-amylphenyl)benzotriazole, in rats (U.S. EPA, 2001). Further studies are needed to clarify the differences in the toxicological profiles between the current study and study on the analogue.

The results of the current study clearly showed sex differences in the toxic susceptibility of rats to HDBB. In males, the development of anemia and histopathologic changes in the liver, heart, kidneys, thyroid, and spleen accompanied with related blood biochemical and organ weight changes were seen. Hypertrophy of hepatocytes, decreased incidence of fatty change of hepatocytes, bile duct proliferation, increase in relative liver weight and serum A/G ratio were noted even at 0.5 mg/kg. Most of the changes were not improved after a 14-day recovery period in the highest dose group. In females, however, no anemic effects of HDBB were observed, and other effects observed in males were noted only at 12.5 mg/kg and more in females. These changes in females mostly recovered after the recovery period. These findings suggest that male rats have a nearly 25 times higher susceptibility to HDBB toxicity than female rats.

Gender-related differences in toxic susceptibility have been documented for some other substances. For example, a recent subchronic toxicity study using F344 rats showed that fluoranthene, a polycyclic aromatic hydrocarbon, had greater effects on males than females (especially on the kidneys) (Knuckles et al., 2004). In contrast, it was reported that female rats exhibited a greater

susceptibility to hypothermic effects and inhibition of hypothalamic cholinesterase by a carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). Such gender-related variation is also reported in humans, mostly for drugs, such as more severe adverse effects with greater improvement in response to antipsychotic drugs such as chlorpromazine and fluspirilene in women (Fletcher et al., 1994; Harris et al., 1995). The various causes of these gender differences are indicated mainly for toxicokinetic determinants. It is well-known that hepatic metabolism differs between the sexes, with males generally having higher activity than females in rats (Gad, 2006). Furthermore, gender differences in membrane transport in various organs of the body including the kidneys, liver, intestine, and brain have emerged relatively recently (Morris et al., 2003). In the case of HDBB, it is difficult to discuss the cause of the gender differences because no other data are available on toxicity, including the toxicokinetics. However, because male rats showed higher susceptibility to various effects of HDBB (on the liver, heart, blood, etc.) consistently, such differences in metabolism or transports between the sexes might increase the blood concentration of causative substances (HDBB or its metabolites) in males.

For gender differences, it goes without saying that sexual hormones play an important role. In fact, Wang et al. (2001) reported that orchidectomy completely abolished the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine. Because testosterone decreased cholinesterase inhibition in gonadectomized males and females, it is apparent that testosterone interferes with the effects of rivastigmine. On the other hand, estrogen has been shown to act as a dopamine antagonist (Fletcher et al., 1994; Harris et al., 1995), which is considered to contribute at least in part to sex differences in response to antipsychotic drugs. It would be interesting to investigate the role of sex steroids in the mediation of sex differences in toxic susceptibility to HDBB, too. For the metabolic enzyme cytochrome P450, involved in the metabolism of many substances, gonadal hormones are known to play an important role in regulating the expression; however, gonadal hormones do not act directly on the liver to confer the sex-dependent pattern, but rather, indirectly via the hypothalamus, which regulates the pituitary and its secretion of the polypeptide hormone, growth hormone (Waxman and Chang, 2005).

Based on the findings of this study, the NOAEL for females was concluded to be 2.5 mg kg^{-1} day^{-1} based on the induction of hypertrophy and increased mitosis of hepatocytes and degeneration and hypertrophy of the myocardium at 12.5 mg/kg. On the other hand, the NOAEL for males could not be determined because hypertrophy and decreased incidence of fatty change of hepatocytes and bile duct proliferation were noted at the lowest dose of 0.5 mg/kg. Considering the toxic effects observed at a relatively low dose and the incomplete recovery, more severe damage by the longer exposure is a concern; therefore, we

are currently performing a 52-week repeated-dose toxicity study to clarify the potential toxic effects of this chemical.

CONCLUSION

The current results showed that the oral administration of HDBB for 28 days principally affected the liver and heart, and male rats were more susceptible to the toxic effects of this chemical than female rats. The NOAEL for repeated-dose toxicity was concluded to be less than 0.5 mg kg^{-1} day^{-1} in male rats and 2.5 mg kg^{-1} day^{-1} in female rats.

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A 52-Week Repeated Dose Toxicity Study of Ultraviolet Absorber 2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole in Rats

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A 52-week repeated dose toxicity study of an ultraviolet absorber, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), was conducted according to OECD TG 452 under GLP. CD(SD)IGS rats were given HDBB by gavage at 0, 0.1, 0.5, or 2.5 mg/kg/day in males and 0, 0.5, 2.5, or 12.5 mg/kg/day in females. No substance-related deaths or clinical signs of toxicity were observed in any group; however, a lowered body weight was found from day 36 to the end of the 52-week administration period at 2.5 mg/kg in males. At the completion of the dosing period, a decrease in red blood cells at 0.5 mg/kg and higher, and in hematocrit at 2.5 mg/kg, was detected in males. Blood biochemical changes, including increases in the levels of alkaline phosphatase and glucose and the A/G ratio, were also found at 0.5 mg/kg and higher in males and at 12.5 mg/kg in females. At necropsy, absolute and relative liver weight was increased at 0.5 mg/kg and higher in males and at 12.5 mg/kg in females. Histopathological changes were observed in the liver; centrilobular hypertrophy of hepatocytes at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females, and altered hepatocellular foci at 0.5 mg/kg and higher, and cystic degeneration and lipofuscin deposition in hepatocytes at 2.5 mg/kg in males. Based on these findings, the no observed adverse effect level was concluded to be 0.1 mg/kg/day in male rats and 2.5 mg/kg/day in female rats.

Keywords Benzotriazole UV absorber, Chronic toxicity, Rat, Gender-related difference.

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INTRODUCTION

Ultraviolet (UV) absorbers are added to plastics to prevent polymer degradation due to UV rays, such as loss of strength, reduced flexibility and electric properties, discoloration, scratching, and loss of gloss (Commerce Online, 2007; Tenkazai.com, 2007). Currently, many kinds of UV absorbers are used: benzotriazoles, benzophenones, salicylates, cyanoacrylates, nickels, triazines, etc. Among them, benzotriazole UV absorbers are known to have the most excellent absorption capacity with a full spectrum of UV absorption and are, therefore, used in a variety of polymers.

2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (CAS No. 3846-71-7; HDBB) is a benzotriazole UV absorber added at ~0.02%–2% mainly to unsaturated polyester resin, polycarbonate, vinyl chloride resin, polyacrylic acid ester, polyacetal, polyolefin, polymethacrylic acid ester, and polyamide (METI, 2006). From these resins, plastic resin products, such as building materials and automobile components, are manufactured. In addition, HDBB is also used in printing or sensitive materials and coating compounds, all intended for UV absorption.

In spite of such widespread use, no reliable data were available on the toxicity of HDBB; therefore, this chemical was selected as an object substance in an existing chemical testing program by the Japanese Government (MHLW, 2003; 2006). Previously, we reported the result of a 28-day repeated dose toxicity study of HDBB conducted under this program (Hirata-Koizumi et al., 2007). In this study, CD(SD)IGS rats were administered HDBB by gavage at a dose of 0.5, 2.5, 12.5, or 62.5 mg/kg/day. As a result, adverse effects, mainly on the liver and heart, were found at all doses in males and at 12.5 mg/kg and higher in females. Anemic changes and histopathological changes in the kidneys and thyroids were also observed at the higher dose. These changes remained after the 14-day recovery period. The no observed adverse effect level (NOAEL) for females was concluded to be 2.5 mg/kg/day based on the induction of hypertrophy and increased mitosis of hepatocytes, and the degeneration and hypertrophy of the myocardium at 12.5 mg/kg. On the other hand, the NOAEL for males could not be determined because hypertrophy and decreased incidence of fatty change of hepatocytes and bile duct proliferation were noted at the lowest dose of 0.5 mg/kg. Considering the toxic effects observed at a relatively low dose and the incomplete recovery, more severe damage induced by longer exposure was a concern; therefore, a chronic toxicity study was performed under the Japanese existing chemical testing program. We here report the details of the results of a 52-week repeated dose toxicity study in rats.

MATERIALS AND METHODS

This study was performed in compliance with the OECD Guideline 452 "Chronic Toxicity Studies" (OECD, 1981) and in accordance with the principles

for Good Laboratory Practice (OECD, 1998; EA, MHW and MITI, 2000) at the Safety Assessment Laboratory, Panapharm Laboratories Co., Ltd. (Kumamoto, Japan).

Chemicals

HDBB was obtained from Shipro Kasei Kaisha, Ltd. (Osaka, Japan). The HDBB (Lot no. S4-034-1) used in this study was 100% pure, based on analysis using liquid chromatography, and it was kept at room temperature. The purity and stability during the study were verified by analysis before and after animal experiments. HDBB was dissolved in corn oil once or twice a week and kept in a dark, cool place until dosing since stability under these conditions was confirmed for up to eight days. The concentrations of formulations were confirmed to be 98.0%–102.0% of the target by analysis using high-performance liquid chromatography (HPLC). All other reagents used in this study were of specific purity grade.

Animals

Crj: CD (SD) IGS rats (SPF, five weeks old) were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). After a seven- or eight-day acclimation, they were subjected to treatment at six weeks of age. Rats found to be in good health were selected and assigned to four groups of 20 males and 20 females by stratified random sampling based on body weight.

All animals were maintained in an air-conditioned room at 21–27°C, with a relative humidity of 47%–60%, a 12-h light/dark cycle, and ventilation with 13–15 air changes/h. They were housed individually, except during the acclimation period, in stainless steel hanger cages. A basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sodium-hypochlorite-added well water were provided *ad libitum*.

This experiment was approved by the Ethical Committee for Animal Experiments of Panapharm Laboratories, Co., Ltd. and performed in accordance with the Guidance for Animal Experiments of Panapharm Laboratories, Co., Ltd.

Experimental Design

Male and female rats were given HDBB once-daily by gavage for 52 weeks at 0 (vehicle control), 0.1, 0.5, or 2.5 mg/kg/day and at 0, 0.5, 2.5, or 12.5 mg/kg/day, respectively. The dosage levels were determined based on the results of our previous 28-day repeated dose toxicity study in rats given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day, in which adverse effects, mainly on the liver and hearts, were found at all doses in males, and at 12.5 mg/kg and more in females (Hirata-Koizumi et al., 2007). The volume of each dose was

adjusted to 5 mL/kg of body weight, based on the latest body weight. At the end of the 13-week administration period, 10 males and 10 females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The remaining animals in all groups (10 rats/sex/dose) were fully examined at the completion of the 52-week administration period.

All animals were observed daily before and after dosing for clinical signs of toxicity. Body weight and food consumption were recorded weekly for the first 13 weeks of the administration period, and once every four weeks for the remainder of the dosing period. At weeks 13 and 52 of the dosing period, fresh urine was collected. It was examined microscopically for urinary sediment and analyzed for dipstick parameters, such as occult blood, pH, protein, glucose, ketone bodies, bilirubin, and urobilinogen. In addition, a 24-h urine sample was also collected for the determination of sodium, potassium, and chlorine levels, color, specific gravity, osmotic pressure, and volume of urine.

Prior to necropsy at the end of the 13- and 52-week dosing periods, blood was collected from the caudal vena cava in the abdomen under deep anesthesia by the intraperitoneal (i.p.) injection of pentobarbital sodium after overnight starvation. One portion of the blood was treated with ethylenediaminetetraacetic acid (EDTA)-2K and examined for hematological parameters, such as red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count, platelet count, reticulocyte count, and differential leukocyte count. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using plasma separated from another blood sample treated with 3.8% sodium citrate. Serum from the remaining portions of blood was analyzed for blood biochemistry (total protein, protein fraction ratio, albumin-globulin (A/G) ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase (ALP)., calcium, inorganic phosphorus, sodium, potassium, and chlorine).

Following the collection of blood, all animals were sacrificed by exsanguination, and organs and tissues of the entire body were macroscopically observed. The brain, pituitary, thymus, thyroids (including parathyroids), heart, lungs (including bronchus), liver, spleen, kidneys, adrenals, testes, epididymides, ovaries, and uterus were then excised and weighed. The trachea, pancreas, lymph nodes (mandibular and mesenteric), tongue, sublingual gland, submandibular gland, parotid gland, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, optic nerve, Harderian gland, spinal cord (pectoral and lumbar part), sciatic nerve, seminal vesicles, prostates, vagina, mammary gland, aorta (thoracic), bone (sternum and femur including bone marrow), skeletal muscle (biceps femoris