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*Original Article*

# A repeated dose 28-day oral toxicity study of β-bromostyrene in rats

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**ABSTRACT** — To obtain information on the possible repeated-dose oral toxicity of β-bromostyrene and its reversibility, Crl: CD (SD) rats were administered β-bromostyrene through gavage at 0, 30, 125, and 500 mg/kg/day once for 28 days, followed by a 14-day recovery period. In the 500 mg/kg group, decrease in spontaneous movement was observed in all males and females on the first dosing day, and one female rat died on Day 3. There were no significant changes in body weight or food consumption. An increase in urine volume and decrease in urine osmolality were observed in males receiving 125 mg/kg and above, and an increase in urine volume was observed in females receiving 500 mg/kg. On blood biochemical examination, increases in total cholesterol, phospholipids, triglycerides, total protein, albumin, inorganic phosphorus, and/or chlorine were observed in the 125 and/or 500 mg/kg groups. Histopathologically, eosinophilic bodies of tubular cells and/or renal tubular degeneration were observed in the kidneys of males in the 125 and 500 mg/kg groups. In the thyroid, hypertrophy of follicular cells was observed in females receiving 125 mg/kg and above and males receiving 500 mg/kg. Furthermore, centrilobular hepatocellular hypertrophy was observed in both sexes receiving 500 mg/kg. These changes observed at the end of the dosing period disappeared or were reduced after the recovery period. Based on these results, the no-observed-adverse-effect-level of β-bromostyrene was judged to be 30 mg/kg/day for both sexes.

**Key words:** β-bromostyrene, CAS No. 103-64-0, OECD TG 407, Repeated dose toxicity, Rat, Gavage

## INTRODUCTION

Safety information on chemicals is necessary for the proper use and management of chemical substances or products containing them. In Japan, the existing chemicals testing program has been conducted by the government. In the program, the Ministry of Health, Labour and Welfare is conducting safety testing and gathering safety information related to health risks on existing chemicals to which humans may be exposed. β-bromostyrene (CAS No. 103-64-0) is a yellow-clear liquid used as an ingredient in mildly fragrant materials, including soap, detergent, creams, lotions, and perfume (HSDB, 1993). Only limited information is available about the toxicity of β-bromostyrene. It has been reported that the oral 50% lethal dose is 1250 mg/kg in rats (HSDB). Since there is insufficient information on its toxicity and no data available on the actual exposure levels at present, this chemical was selected as an object substance in the existing chemical testing program by the Japanese government. In this paper, we report the result of a 28-day repeated oral administration study of β-bromostyrene.

## MATERIALS AND METHODS

The present study was conducted at BoZo Research Center Inc. (Shizuoka, Japan). This study was designed to meet the Japanese Test Guidelines for Toxicology Studies issued by "Notification test methods of New Chemical Substances" (Yakushokuhatsu No. 1121002, Seikyoku No. 2, Kanpokihatsu No. 031121002, last revision November 20, 2006) and OECD Guideline for the Testing of Chemicals (TG 407, adopted on July 27, 1995), and was conducted in compliance with the Good

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Laboratory Practice Standards criteria for test facilities for carrying out tests on new chemical substances, etc. in Japan (Yakushokuhatsu No. 1121003, Seikyoku No. 3, and Kanpokihatsu No. 031121004, last revision April 1, 2005). The use and care of animals complied with the Act on Welfare and Management of Animals (Japanese Animal Welfare Law, Act No. 105, last revision June 22, 2005), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Announcement No. 88, Ministry of the Environment, Japan, April 28, 2006), and Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006).

### Test substance and reagent

β-bromostyrene [lot no. TEYUC, purity 99.6% (cis- and trans-mixture), yellow-clear liquid, CAS No. 103-64-0] was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and kept in a test substance storage room (light shielding and moisture prevention) of the testing facility at approximately 3-9°C. Corn oil (lot no. WKJ3948) as a vehicle was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

### Animals and husbandry

Specific-pathogen-free Sprague-Dawley rats [Crl: CD (SD)] at 5 weeks of age were purchased from Atsugi Breeding Center of Charles River Japan, Inc. (Kanagawa, Japan). Forty-seven males and 47 females were obtained and individually identified using ear tags. Rats were quarantined and acclimatized to the testing environment for 7 days and assigned to each dose group by stratified random sampling based on body weight. Administration of the test substance was initiated at 6 weeks of age. Body weight ranges for males and females upon initiation of treatment were 182-216 g and 145-171 g, respectively. Animals were individually housed in wire-mesh steel bracket cages (W 250 × D 350 × H 200 mm) and kept in an environmentally-controlled room: temperature, 21-23°C; humidity, 49-66%; ventilation, 10-15 times/hr; and lighting, 12 hr per /day (light on/off, 7:00/19:00). The animals were fed a pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and given tap water through bottle *ad libitum*.

### Selection of dose levels

Dose levels of β-bromostyrene were selected based on results obtained from a 14-day dose range-finding study using the same strain of rats (five males and five females per group) at dose levels of 0 (corn oil only), 100, 300, and 1000 mg/kg/day. In the dose range-finding study, all males and females in the 1000 mg/kg group died. Increases in relative liver and kidney weights were observed in the 300 mg/kg group. Therefore, in the present study, the high dose was set at 500 mg/kg/day, and middle and low doses were set at 125 and 30 mg/kg/day, respectively, using common ratio 4.

### Experimental design

Rats were administered β-bromostyrene by gavage once daily at 0 (vehicle control), 30, 125, and 500 mg/kg/day for 28 days. There were 12 rats/sex/dose in the 0 and 500 mg/kg groups and 6 rats/sex/dose in the 30 and 125 mg/kg groups; the dosing volume was 5 mL/kg body weight. On the day after the last dosing, six males and six females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weight, and macroscopic and microscopic findings (main group). The respective remaining 6 rats/sex at 0 and 500 mg/kg were kept without treatment for 14 days as a recovery period and then fully examined (recovery group).

### Daily observation, functional observation battery, body weight, and food and water consumption

All animals were observed, in their cages, for clinical signs of toxicity 2-3 times daily during the dosing period and once daily during the recovery period. Detailed clinical observations, including observations in the home cage, during handling, and outside of the home cage in an open field, were conducted before the start of dosing and once a week during the dosing and recovery periods. At the end of the dosing and recovery periods, functional observations, including auditory, approach, touch, and tail pinch responses, pupillary and aerial righting reflexes, and landing foot splay, were performed. In addition, grip strengths (fore and hindlimb) were measured using a CPU gauge (model-9502A, Aikoh Engineering Co., Ltd., Osaka, Japan), and motor activity was recorded at 10 min intervals for 1 hr by an activity monitoring system (model NS-AS01, Neuroscience, Inc., Tokyo, Japan). Body weight was recorded before dosing on Days 1, 4, 7, 10, 14, 17, 21, 24, and 28 of the dosing period and on Days 1, 3, 7, 10, and 14 of the recovery period. Food consumption was measured on the same days as body weights. Water consumption was recorded during Week 4 of the dosing period and Week 2 of the recovery period.

### Urinalysis, hematology, and clinical biochemistry

Urinalysis was conducted during Week 4 in the dosing period and Week 2 in the recovery period. Urine was

collected for 4 hrs under fasting conditions with water *ad libitum* and analyzed using an AUTION MINI™ AM-4290 (Arkray Inc., Kyoto, Japan) for dipstick parameters, such as pH, proteins, ketone bodies, glucose, occult blood, bilirubin, urobilinogen, color, sediments, and volume. Urine volume and osmolality were measured using an automatic osmometer (Auto & Stat OM-6030, Arkray Inc., Kyoto, Japan) using a 20-hr urine sample collected with food and water *ad libitum*.

The day after the end of the dosing and recovery periods, blood was collected from the abdominal aorta under deep anesthesia after overnight starvation. One portion of the blood was treated with EDTA-2K and examined using an Advia 120 Hematology System (Siemens Medical Solutions Diagnostics, New York, USA) for hematologic parameters, such as red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte, platelet (PLT), white blood cell (WBC), and differential leukocyte count [lymphocyte (LYMP), neutrophil (NEUT), eosinophil (EOS), basophil (BASO), monocyte (MONO), and large unstained cell (LUC)]. Another blood sample was treated with sodium citrate and analyzed using a coagulometer ACL 100 (Instrumentation Laboratory, Massachusetts, USA) for blood clotting parameters, such as prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen level (FIB).

Serum from the remaining portion of blood was analyzed for alkaline phosphatase (ALP), total cholesterol (T-CHO), triglyceride (TG), phospholipid (PL), total bilirubin (T-BIL), glucose (GLU), blood urea nitrogen (BUN), creatinine (CRNN), sodium (Na), potassium (K), chlorine (Cl), calcium (Ca), inorganic phosphorus (P), total protein (TP), albumin (ALB), and albumin/globulin (A/G) ratio. Plasma isolated from heparinized blood was analyzed for aspartate and alanine aminotransferases (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and γ-glutamyl transpeptidase (γ-GTP). Items excluding electrolytes were analyzed using a clinical chemistry automatic analyzer (TBA-120FR, Toshiba Corporation, Tokyo, Japan) and electrolytes were analyzed by an automatic analyzer (PVA-αII, Analytical Instruments, Inc., Massachusetts, USA).

### Organ weights, gross necropsy, and histopathology

After blood collection, all animals were sacrificed by exsanguination, and the organs and tissues of the whole body, including external surfaces, head, breast, and abdomen, were observed macroscopically. Next, the brain, adrenals, thymus, spleen, heart, liver, kidneys, testes, epididymides, ovaries, and uterus were removed and weighed. In addition, relative organ weights were calculated from organ/body weight ratios.

The cerebrum, cerebellum, spinal cord (chest), sciatic nerve, pituitary gland, thyroid, parathyroids, adrenal glands, thymus, spleen, submandibular lymph nodes, mesenteric lymph nodes, heart, trachea, lung (including bronchial), stomach, duodenum, jejunum, ileum (including Peyer's patches), cecum, colon, rectum, liver, kidneys, urinary bladder, testes, epididymides, prostate, ovaries, uterus, sternum (including bone marrow), femur (including bone marrow), and femoral skeletal muscle were fixed in 10% phosphate-buffered formalin. The eyeballs and optic nerves were fixed in phosphate-buffered 3 vol% glutaraldehyde/2.5 vol% formalin, and the testes and epididymides were fixed in Bouin's solution.

Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin. In the control and high dose groups sacrificed at the end of the dosing period, all preserved organs were examined under a light microscope. If treatment-related histopathological changes were found, the same tissues were examined for low and middle dose groups and the recovery group.

### Data analysis

Parametric data, such as quantitative data in open field observation, functional observation and urinalysis, grip strengths, motor activity, body weight, food and water consumption, hematological and blood biochemistry findings, and organ weights, were analyzed using Bartlett's test for homogeneity of distribution. The Dunnett's multiple comparison test and the Dunnett's-type mean rank sum test were conducted for homogenous and non-homogenous distribution, respectively to compare the control and individual treatment groups. Parametric data obtained during or after the recovery period were analyzed by *F*-test for homogeneity of distribution. For comparison, the Student's *t*-test and the Aspin-Welch's *t*-test were conducted for homogenous and non-homogenous distribution, respectively, (Snedecor and Cochran, 1989; Dunnett, 1955, 1964; Sakuma, 1977, 1981).

## RESULTS

### General clinical observations

No abnormal clinical signs were observed in either sex receiving 30 or 125 mg/kg during the dosing period. One female rat receiving 500 mg/kg was found dead

on Day 3. In this rat, decrease in spontaneous movement was observed only on the first dosing day. Decreases in spontaneous movement were also observed in all other male and female rats receiving 500 mg/kg on the first dosing day; however, no abnormalities were observed in the general conditions thereafter during the dosing period. No clinical signs were observed in any animal during the recovery period.

### Detailed clinical and functional observations

Detailed clinical observations: In the open field observation, a significant increase in rearing counts was observed in males receiving 30 mg/kg only during Week 1 of the dosing period; this was not observed in the higher dose groups. A significantly low number of rearing counts was observed in females receiving 500 mg/kg during Week 4, but this value was equivalent to those during Weeks 1-3 in the same group. During handling, slight salivation was observed in four males and one female during Week 3 and in three males and two females during Week 4 in the 500 mg/kg group. There were no abnormal or significant changes in recovery group rats.

Functional observations: No significant changes were observed in any parameter for either sex receiving the test substance during Week 4 of the dosing period. A significant decrease in landing foot splay was observed in males receiving 500 mg/kg during Week 2 of the recovery period; however, it was determined to be incidental because this sign was not observed during Week 4 of the dosing period.

Grip strength: A significant increase in hindlimb grip strength was observed in females receiving 125 mg/kg during Week 4 of the dosing period. However, this was not observed in the high dose group. A significant decrease in forelimb grip strength was observed in males receiving 500 mg/kg during Week 2 of the recovery period, but this change was not observed during Week 4 of the dosing period.

Motor activity: No significant change was observed in any male or female rats receiving the test substance during Week 4 of the dosing period. During Week 2 of the recovery period, a significant decrease was observed in males receiving 500 mg/kg only 40-50 min after the start of measurement.

### Body weight, food consumption, and water consumption

Body weights in females receiving 125 mg/kg were significantly higher at Days 17-24 during the dosing period, but no significant differences were found in the 500 mg/kg groups throughout the study. In the 500 mg/kg group, food consumption significantly decreased for both sexes at Day 4 of the dosing period and in females at Days 7 and 14 of the recovery period. On the other hand, food consumption significantly increased in females at Days 7-21 of the dosing period in the 125 mg/kg group and Days 7, 14-21, and 28 of the dosing period in the 500 mg/kg group. A significant decrease in water consumption was observed in females receiving 125 mg/kg during Week 4 of the dosing period; however, this change was not observed in the high dose group. No significant differences were seen with water consumption for either sex in the recovery group.

### Urinalysis

During Week 4 of the dosing period, significant increases in urine volume were observed in males receiving 125 and 500 mg/kg (12.1 ± 3.0 and 13.1 ± 4.4 mL/24 hr, respectively, versus 7.4 ± 3.6 mL/24 hr for control) and in females receiving 500 mg/kg (10.9 ± 3.5 mL/24 hr versus 6.4 ± 3.2 mL/24 hr for control). A significant decrease in urine osmolality was also observed in males receiving 125 and 500 mg/kg (1783 ± 359 and 1665 ± 328 mOsm/kg, respectively, versus 2194 ± 355 mOsm/kg for control). In the sediments, small round epithelial cells were observed in 5/12 males and 1/11 females receiving 500 mg/kg, and this change increased in males compared with the control group. No significant differences were seen in urine volume, osmolality, or qualitative measurements for either sex compared with the control groups during Week 2 of the recovery period.

### Hematology

Hematological results are summarized in Table 1. At the end of the dosing period, significant decreases in MCH were observed in males receiving 30 and 500 mg/kg, and significant decreases in MCH concentration were observed in both sexes receiving 500 mg/kg. A significant increase in reticulocytes was also found in females receiving 500 mg/kg. However, these changes were slight, and no clear changes were observed in RBC or HGB. Other significant changes were a reduction in APTT and an increase in FIB in females receiving 125 mg/kg, but these changes were not observed at 500 mg/kg. Therefore, these changes were determined to be incidental. At the end of the recovery period, the only significant changes observed were a decrease in EOSs in males and an increase in MONOs in females. However, these changes were not observed at the end of the dosing period; therefore, these changes were determined to be incidental.

**Table 1.** Hematological values in the repeated dose 28-day oral toxicity study of β-bromostyrene in rats.

| Dose (mg/kg/day) | At the end of the dosing period | | | | At the end of the recovery period | |
|---|---|---|---|---|---|---|
| | 0 | 30 | 125 | 500 | 0 | 500 |
| Males | | | | | | |
| No. of animals | 6 | 6 | 6 | 6 | 6 | 6 |
| RBC ($\times10^4$/μL) | 795 ± 31 | 817 ± 29 | 816 ± 31 | 831 ± 38 | 855 ± 15 | 873 ± 39 |
| HGB (g/dL) | 16.1 ± 0.5 | 15.8 ± 0.5 | 16.0 ± 0.6 | 15.9 ± 0.5 | 15.9 ± 0.4 | 16.1 ± 0.4 |
| HCT (%) | 43.7 ± 1.7 | 43.5 ± 1.4 | 43.9 ± 1.6 | 44.2 ± 1.9 | 44.2 ± 1.1 | 44.9 ± 1.2 |
| MCV (fL) | 55.0 ± 1.4 | 53.2 ± 0.9 | 53.9 ± 1.5 | 53.2 ± 1.2 | 51.7 ± 1.2 | 51.6 ± 2.3 |
| MCH (pg) | 20.2 ± 0.5 | 19.3 ± 0.4* | 19.6 ± 0.6 | 19.2 ± 0.6** | 18.5 ± 0.4 | 18.5 ± 0.8 |
| MCHC (g/dL) | 36.8 ± 0.3 | 36.3 ± 0.3 | 36.4 ± 0.2 | 36.1 ± 0.6* | 35.9 ± 0.4 | 35.9 ± 0.3 |
| Reticulocyte (%) | 2.1 ± 0.5 | 1.9 ± 0.1 | 2.0 ± 0.2 | 1.9 ± 0.3 | 1.8 ± 0.4 | 1.8 ± 0.4 |
| PLT ($\times10^4$/μL) | 130.8 ± 6.2 | 130.1 ± 8.5 | 121.7 ± 13.5 | 122.3 ± 16.2 | 114.2 ± 13.8 | 128.3 ± 11.8 |
| PT (sec) | 14.3 ± 1.0 | 13.7 ± 1.0 | 13.6 ± 1.0 | 15.8 ± 1.5 | 15.9 ± 3.3 | 16.1 ± 1.5 |
| APTT (sec) | 22.8 ± 3.1 | 20.8 ± 1.9 | 20.3 ± 1.8 | 23.7 ± 2.2 | 24.7 ± 2.1 | 23.9 ± 1.6 |
| FIB (mg/dL) | 332 ± 25 | 334 ± 26 | 332 ± 31 | 360 ± 26 | 292 ± 27 | 331 ± 38 |
| WBC ($\times10^2$/μL) | 104.5 ± 21.1 | 100.0 ± 12.4 | 118.1 ± 26.6 | 119.3 ± 10.2 | 89.7 ± 19.5 | 110.4 ± 31.9 |
| Differential leukocyte count (%) | | | | | | |
| LYMP | 79.2 ± 3.3 | 82.1 ± 3.4 | 78.7 ± 7.8 | 80.0 ± 3.7 | 79.3 ± 2.9 | 77.2 ± 6.5 |
| NEUT | 17.7 ± 3.1 | 14.7 ± 3.3 | 17.7 ± 7.5 | 16.0 ± 3.9 | 16.6 ± 3.0 | 19.4 ± 6.1 |
| EOS | 0.7 ± 0.2 | 1.0 ± 0.5 | 0.8 ± 0.3 | 0.7 ± 0.2 | 1.4 ± 0.4 | 0. 9 ± 0.2** |
| BASO | 0.3 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| MONO | 1.5 ± 0.5 | 1.4 ± 0.2 | 1.9 ± 0.8 | 2.2 ± 0.4 | 1.9 ± 0.4 | 1.7 ± 0.6 |
| LUC | 0.5 ± 0.2 | 0.6 ± 0.2 | 0.6 ± 0.2 | 0.8 ± 0.4 | 0.5 ± 0.2 | 0.5 ± 0.2 |
| Females | | | | | | |
| No. of animals | 6 | 6 | 6 | 6 | 6 | 5 |
| RBC ($\times10^4$/μL) | 802 ± 31 | 796 ± 38 | 794 ± 37 | 827 ± 46 | 825 ± 21 | 861 ± 51 |
| HGB (g/dL) | 15.7 ± 0.2 | 15.7 ± 0.5 | 15.6 ± 0.5 | 15.7 ± 0.4 | 15.8 ± 0.5 | 15.9 ± 0.7 |
| HCT (%) | 42.0 ± 0.5 | 42.0 ± 1.5 | 42.0 ± 1.7 | 43.3 ± 1.1 | 42.5 ± 1.1 | 43.2 ± 2.1 |
| MCV (fL) | 52.5 ± 2.1 | 52.7 ± 1.3 | 53.0 ± 1.0 | 52.5 ± 1.8 | 51.6 ± 1.5 | 50.2 ± 0.8 |
| MCH (pg) | 19.6 ± 0.7 | 19.7 ± 0.6 | 19.7 ± 0.6 | 19.0 ± 1.0 | 19.1 ± 0.6 | 18.5 ± 0.4 |
| MCHC (g/dL) | 37.3 ± 0.4 | 37.5 ± 0.6 | 37.2 ± 0.5 | 36.2 ± 0.6** | 37.1 ± 0.4 | 36.8 ± 0.4 |
| Reticulocyte (%) | 1.2 ± 0.3 | 1.5 ± 0.3 | 1.4 ± 0.4 | 1.7 ± 0.2* | 1.3 ± 0.4 | 1.2 ± 0.4 |
| PLT ($\times10^4$/μL) | 142.9 ± 14.1 | 136.9 ± 9.8 | 138.4 ± 9.9 | 130.6 ± 9.2 | 137.2 ± 14.4 | 131.9 ± 8.7 |
| PT (sec) | 11.5 ± 0.4 | 11.3 ± 0.6 | 11.1 ± 0.5 | 11.6 ± 0.7 | 12.2 ± 0.5 | 12.1 ± 0.7 |
| APTT (sec) | 17.7 ± 1.6 | 16.2 ± 1.3 | 15.4 ± 1.4* | 16.1 ± 1.8 | 18.2 ± 1.7 | 20.6 ± 2.0 |
| FIB (mg/dL) | 220 ± 19 | 234 ± 20 | 256 ± 12** | 243 ± 18 | 214 ± 15 | 246 ± 40 |
| WBC ($\times10^2$/μL) | 78.5 ± 9.4 | 83.2 ± 16.5 | 82.3 ± 10.7 | 91.5 ± 18.6 | 74.6 ± 19.7 | 87.3 ± 23.5 |
| Differential leukocyte count (%) | | | | | | |
| LYMP | 81.9 ± 4.8 | 75.5 ± 8.1 | 79.9 ± 8.9 | 78.5 ± 8.7 | 77.9 ± 6.2 | 75.8 ± 4.6 |
| NEUT | 14.3 ± 4.7 | 20.2 ± 8.4 | 16.1 ± 8.5 | 17.1 ± 8.8 | 18.4 ± 5.9 | 19.8 ± 5.5 |
| EOS | 1.2 ± 0.4 | 1.5 ± 0.7 | 1.1 ± 0.3 | 1.3 ± 0.6 | 1.4 ± 0.6 | 1.4 ± 0.8 |
| BASO | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.0 |
| MONO | 1.7 ± 0.7 | 2.0 ± 0.7 | 2.0 ± 0.9 | 2.2 ± 1.4 | 1.4 ± 0.3 | 2.3 ± 0.7* |
| LUC | 0.7 ± 0.2 | 0.6 ± 0.3 | 0.7 ± 0.4 | 0.6 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.2 |

Values are expressed as the mean ± standard deviation. *$P < 0.05$ and **$P < 0.01$ versus control.
RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; PT, prothrombin time; APTT, activated partial thromboplastin time; FIB, fibrinogen; WBC, white blood cells; LYMP, lymphocytes; NEUT, neutrophils; EOS, eosinophils; BASO, basophils; MONO, monocytes; LUC, large unstained cells.

**Table 2.** Clinical biochemistry values of the repeated dose 28-day oral toxicity study of β-bromostyrene in rats.

| Dose (mg/kg/day) | At the end of the dosing period | | | | At the end of the recovery period | |
|---|---|---|---|---|---|---|
| | 0 | 30 | 125 | 500 | 0 | 500 |
| Males | | | | | | |
| No. of animals | 6 | 6 | 6 | 6 | 6 | 6 |
| AST (IU/L) | 65 ± 3 | 57 ± 4 | 56 ± 7* | 57 ± 8* | 66 ± 3 | 64 ± 6 |
| ALT (IU/L) | 28 ± 3 | 27 ± 3 | 24 ± 3 | 23 ± 4* | 30 ± 3 | 27 ± 3 |
| LDH (IU/L) | 73 ± 19 | 60 ± 11 | 52 ± 8* | 75 ± 17 | 51 ± 6 | 54 ± 6 |
| γ-GTP (IU/L) | 1 ± 1 | 1 ± 0 | 1 ± 0 | 1 ± 0 | 1 ± 0 | 1 ± 0 |
| ALP (IU/L) | 704 ± 186 | 647 ± 112 | 667 ± 95 | 645 ± 116 | 530 ± 100 | 542 ± 76 |
| T-CHO (mg/dL) | 52 ± 13 | 54 ± 12 | 67 ± 12 | 67 ± 13 | 62 ± 16 | 69 ± 17 |
| TG (mg/dL) | 50 ± 33 | 71 ± 26 | 72 ± 35 | 54 ± 18 | 45 ± 19 | 52 ± 18 |
| PL (mg/dL) | 88 ± 21 | 93 ± 14 | 110 ± 19 | 108 ± 15 | 96 ± 15 | 108 ± 20 |
| T-BIL (mg/dL) | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| GLU (mg/dL) | 137 ± 17 | 142 ± 7 | 137 ± 11 | 137 ± 12 | 129 ± 11 | 127 ± 21 |
| BUN (mg/dL) | 12 ± 1 | 13 ± 1 | 11 ± 1 | 11 ± 1 | 14 ± 1 | 13 ± 1 |
| CRNN (mg/dL) | 0.25 ± 0.03 | 0.22 ± 0.03 | 0.22 ± 0.01 | 0.21 ± 0.03* | 0.25 ± 0.02 | 0.25 ± 0.01 |
| Na (mmol/L) | 143 ± 1 | 143 ± 1 | 143 ± 2 | 143 ± 1 | 145 ± 1 | 145 ± 1 |
| K (mmol/L) | 4.7 ± 0.2 | 4.8 ± 0.2 | 4.5 ± 0.2 | 4.9 ± 0.2 | 4.6 ± 0.2 | 4.6 ± 0.2 |
| Cl (mmol/L) | 112 ± 2 | 110 ± 2 | 111 ± 2 | 111 ± 1 | 110 ± 1 | 110 ± 2 |
| Ca (mg/dL) | 9.5 ± 0.1 | 9.7 ± 0.3 | 9.9 ± 0.2* | 9.9 ± 0.2** | 9.7 ± 0.2 | 9.7 ± 0.2 |
| P (mg/dL) | 7.8 ± 0.4 | 8.4 ± 0.6 | 8.3 ± 0.3 | 9.7 ± 0.8** | 7.4 ± 0.6 | 7.7 ± 0.3 |
| TP (g/dL) | 5.9 ± 0.1 | 6.0 ± 0.2 | 5.9 ± 0.2 | 6.4 ± 0.3** | 6.3 ± 0.3 | 6.3 ± 0.3 |
| ALB (g/dL) | 2.7 ± 0.1 | 2.8 ± 0.1 | 2.8 ± 0.1 | 2.9 ± 0.1** | 2.9 ± 0.1 | 2.9 ± 0.1 |
| A/G | 0.87 ± 0.04 | 0.89 ± 0.05 | 0.88 ± 0.05 | 0.85 ± 0.04 | 0.86 ± 0.08 | 0.86 ± 0.05 |
| Females | | | | | | |
| No. of animals | 6 | 6 | 6 | 6 | 6 | 5 |
| AST (IU/L) | 63 ± 6 | 61 ± 7 | 56 ± 3 | 55 ± 8 | 61 ± 7 | 57 ± 6 |
| ALT (IU/L) | 25 ± 3 | 24 ± 5 | 21 ± 3 | 19 ± 3 | 25 ± 5 | 22 ± 2 |
| LDH (IU/L) | 60 ± 17 | 58 ± 17 | 60 ± 16 | 57 ± 12 | 52 ± 12 | 52 ± 10 |
| γ-GTP (IU/L) | 1 ± 0 | 1 ± 1 | 1 ± 0 | 2 ± 1 | 1 ± 0 | 1 ± 0 |
| ALP (IU/L) | 334 ± 90 | 407 ± 166 | 377 ± 66 | 362 ± 114 | 304 ± 63 | 241 ± 46 |
| T-CHO (mg/dL) | 41 ± 7 | 53 ± 19 | 72 ± 19* | 92 ± 18** | 63 ± 8 | 83 ± 23 |
| TG (mg/dL) | 10 ± 4 | 14 ± 8 | 18 ± 6 | 19 ± 5* | 17 ± 7 | 27 ± 8* |
| PL (mg/dL) | 77 ± 11 | 91 ± 23 | 113 ± 23* | 141 ± 23** | 113 ± 16 | 137 ± 20 |
| T-BIL (mg/dL) | 0.1 ± 0.0 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| GLU (mg/dL) | 98 ± 11 | 110 ± 19 | 115 ± 10 | 114 ± 9 | 107 ± 9 | 117 ± 18 |
| BUN (mg/dL) | 15 ± 1 | 16 ± 3 | 14 ± 2 | 12 ± 2 | 18 ± 2 | 16 ± 3 |
| CRNN (mg/dL) | 0.28 ± 0.03 | 0.28 ± 0.05 | 0.27 ± 0.03 | 0.23 ± 0.04 | 0.31 ± 0.03 | 0.27 ± 0.05 |
| Na (mmol/L) | 142 ± 1 | 142 ± 1 | 142 ± 1 | 141 ± 1 | 143 ± 1 | 143 ± 1 |
| K (mmol/L) | 4.9 ± 0.2 | 4.7 ± 0.3 | 4.7 ± 0.3 | 4.6 ± 0.2 | 4.6 ± 0.2 | 4.3 ± 0.3 |
| Cl (mmol/L) | 113 ± 2 | 114 ± 1 | 114 ± 1 | 116 ± 1* | 113 ± 1 | 111 ± 2 |
| Ca (mg/dL) | 9.8 ± 0.3 | 9.7 ± 0.1 | 9.8 ± 0.4 | 10.1 ± 0.3 | 9.9 ± 0.3 | 10.1 ± 0.3 |
| P (mg/dL) | 7.7 ± 0.5 | 7.7 ± 0.4 | 7.5 ± 0.5 | 7.2 ± 1.0 | 7.0 ± 0.3 | 7.3 ± 0.7 |
| TP (g/dL) | 6.1 ± 0.3 | 6.1 ± 0.2 | 6.1 ± 0.3 | 6.5 ± 0.2* | 6.5 ± 0.3 | 6.7 ± 0.1 |
| ALB (g/dL) | 2.9 ± 0.2 | 2.9 ± 0.1 | 3.0 ± 0.2 | 3.1 ± 0.1 | 3.1 ± 0.1 | 3.2 ± 0.1 |
| A/G | 0.92 ± 0.04 | 0.91 ± 0.07 | 0.95 ± 0.06 | 0.90 ± 0.04 | 0.91 ± 0.02 | 0.90 ± 0.05 |

Values are expressed as the mean ± standard deviation. *$P < 0.05$ and **$P < 0.01$ versus control.

AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; T-CHO, total cholesterol; TG, triglycerides; PL, phospholipids; T-BIL, total bilirubin; GLU, glucose; BUN, blood urea nitrogen; CRNN, creatinine; TP, total protein; ALB, albumin; A/G, albumin/globulin ratio.

### Clinical biochemistry

Clinical biochemistry results are summarized in Table 2. At the end of the dosing period, T-CHO and PL levels were significantly increased in rats receiving 125 mg/kg and above, and TG levels were also significantly increased in females receiving 500 mg/kg. Slight but significant decreases were found in AST activity at doses of 125 and 500 mg/kg, in ALT activity at 500 mg/kg, and in LDH activity at 125 mg/kg in males. In the 500 mg/kg group, there was a significant increase in TP levels for either sex, a significant increase in ALB levels in males, and a significant decrease in CRNN in males. Furthermore, significant increases were observed in Cl in females and P in males receiving 500 mg/kg, as well as Ca in males receiving 125 and 500 mg/kg. After the recovery period, TG levels remained significantly high in females in the 500 mg/kg group, but other changes observed at the end of the dosing period were not detected.

### Organ weights

Significant effects by the test substance were observed in the liver and kidneys at the end of the dosing period. Increases were found in absolute and relative liver weights at 500 mg/kg in both sexes along with an increase in absolute liver weight at 125 mg/kg in females, an increase in relative liver weight at 125 mg/kg in males, increases in absolute and relative kidney weights at 500 mg/kg in males, and an increase in relative kidney weights at 500 mg/kg in females (Table 3). The significant increase in relative liver weight remained after the recovery period in females receiving 500 mg/kg. Other significant changes were decreases in relative brain weight at 125 mg/kg in females, relative spleen weight at 30 mg/kg in males, and absolute testes weight at 500 mg/kg at the end of dosing period, as well as an increase in relative heart weight at 500 mg/kg in females at the end of the recovery period. However, these changes were slight and/or lacked dose-dependency.

### Gross necropsy

In the female rat which received 500 mg/kg that was found dead on Day 3 of the dosing period, there were excess fluids in the abdominal and thoracic cavities, an enlarged liver, and dark red foci in the glandular stomach. In animals sacrificed as scheduled, enlargement of the liver was observed in three males and two females receiving 500 mg/kg at the end of the dosing period and one male receiving 500 mg/kg at the end of the recovery period. Other findings included dark red foci in the lung in one control female and one male receiving 125 mg/kg, dark red foci in the glandular stomach in one female each receiving 125 and 500 mg/kg, and unilateral small thyroids in one female each receiving 30 and 500 mg/kg at the end of the dosing period.

### Histopathology

In the dead 500 mg/kg group female, atrophy of Peyer's patch in the ileum, dilation of the renal tubes with centrilobular necrosis and congestion in the liver, focal hemorrhage and accumulation of foamy cells in the lung, atrophy of the mesenteric and submandibular lymph nodes, an increase in hematopoiesis and atrophy of white pulp in the spleen, erosion in the glandular stomach, atrophy of the thymus, and a remnant of ultimobranchial bodies in the thyroid were observed.

Histopathological changes observed in other animals are summarized in Tables 4 and 5. Significant effects of the test substance were observed in the liver and thyroids of both sexes and kidneys of males at the end of the dosing period. In the kidneys, a minimal to mild degree of eosinophilic bodies in tubular cells was observed in two males receiving 125 mg/kg and all males receiving 500 mg/kg. Mild degeneration of renal tubular and minimal hyaline casts was also observed in males in the 500 mg/kg group. In the liver, minimal to mild centrilobular hypertrophy of hepatocytes was observed in all males and females receiving 500 mg/kg. In the thyroids, minimal hypertrophy of follicular cells was observed in one female receiving 125 mg/kg and two males and five females receiving 500 mg/kg.

At the end of the recovery period, significant effects of the test substance were observed in the kidneys and thyroids of males. A minimal degree of eosinophilic bodies in renal tubular cells was observed in three males at 500 mg/kg. Minimal regeneration of renal tubules was observed in three males of the control group and four males of 500 mg/kg group, and mild regeneration was observed in two males of the 500 mg/kg group; the incidence and severity of regeneration were slightly increased in the 500 mg/kg group. Minimal hypertrophy of follicular cells in the thyroid was observed in one male at 500 mg/kg. Other changes at the end of the dosing and/or recovery periods observed in the heart, rectum, kidneys, liver, prostate, skeletal muscle, spleen, stomach, thyroid, and urinary bladder were considered to be incidental findings due to the apparent situation or histopathological properties.

## DISCUSSION

This study examined the toxicity of β-bromostyrene and its reversibility. Crl: CD (SD) rats were administered

**Table 3.** Absolute and relative organ weights in the repeated dose 28-day oral toxicity study of β-bromostyrene in rats.

| Item | Dose (mg/kg/day) | At the end of the dosing period | | | | At the end of the recovery period | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | Females | | Males | | Females | |
| | | Liver | Kidney (R + L) | Liver | Kidney (R + L) | Liver | Kidney (R + L) | Liver | Kidney (R + L) |
| Absolute (g) | 0 | 10.50 ± 2.05 | 2.53 ± 0.25 | 6.26 ± 0.76 | 1.67 ± 0.15 | 11.80 ± 1.27 | 2.97 ± 0.37 | 6.29 ± 0.62 | 1.77 ± 0.11 |
| | 30 | 12.02 ± 1.31 | 2.84 ± 0.33 | 6.69 ± 0.74 | 1.71 ± 0.21 | - | - | - | - |
| | 125 | 12.08 ± 1.45 | 2.88 ± 0.25 | 7.42 ± 0.45* | 1.91 ± 0.18 | - | - | - | - |
| | 500 | 14.83 ± 1.49** | 3.26 ± 0.34** | 9.37 ± 0.79** | 1.85 ± 0.13 | 12.06 ± 1.85 | 2.98 ± 0.26 | 7.38 ± 1.10 | 1.89 ± 0.28 |
| Relative (g/100 g BW) | 0 | 2.94 ± 0.20 | 0.72 ± 0.07 | 2.83 ± 0.19 | 0.76 ± 0.04 | 2.83 ± 0.13 | 0.71 ± 0.05 | 2.55 ± 0.10 | 0.72 ± 0.04 |
| | 30 | 3.26 ± 0.15 | 0.77 ± 0.05 | 2.96 ± 0.22 | 0.76 ± 0.09 | - | - | - | - |
| | 125 | 3.32 ± 0.29* | 0.79 ± 0.04 | 3.11 ± 0.22 | 0.80 ± 0.06 | - | - | - | - |
| | 500 | 4.31 ± 0.24** | 0.95 ± 0.08** | 4.34 ± 0.23** | 0.86 ± 0.07* | 3.00 ± 0.22 | 0.75 ± 0.08 | 3.10 ± 0.31* | 0.79 ± 0.09 |

Values are expressed as the mean ± standard deviation of six rats. *$P < 0.05$ and **$P < 0.01$ versus control.
BW, body weight; R, right; L, left.

**Table 4.** Histopathological findings in the repeated dose 28-day oral toxicity study of β-bromostyrene in rats at the end of the dosing period.

| Organs / Findings | Sex | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Dose (mg/kg/day) | 0 | 30 | 125 | 500 | 0 | 30 | 125 | 500 |
| | Number | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Heart | | | | | | | | | |
| Focal myocarditis | Minimal | 1 | - | - | 0 | 0 | - | - | 0 |
| Intestine, rectum | | | | | | | | | |
| Submucosal cell infiltration | Minimal | 1 | - | - | 0 | 0 | - | - | 0 |
| Kidney | | | | | | | | | |
| Tubular regeneration | Minimal | 2 | 3 | 2 | 3 | 1 | - | - | 2 |
| Eosinophilic body in tubular cells | Minimal | 0 | 0 | 2 | 0 | 0 | - | - | 0 |
| | Mild | 0 | 0 | 0 | 6 | 0 | - | - | 0 |
| Hyaline cast | Minimal | 0 | 0 | 0 | 3 | 1 | - | - | 0 |
| Interstitial mineralization | Minimal | 1 | 5 | 4 | 4 | 4 | - | - | 2 |
| Interstitial cell infiltration | Minimal | 2 | 1 | 1 | 3 | 3 | - | - | 0 |
| Tubular degeneration | Mild | 0 | 0 | 0 | 2 | 0 | - | - | 0 |
| Liver | | | | | | | | | |
| Periportal vacuolation of hepatocytes | Minimal | 1 | 0 | 0 | 0 | 1 | 2 | 1 | 1 |
| | Mild | 0 | 0 | 0 | 0 | 3 | 2 | 0 | 1 |
| Extramedullary hematopoiesis | Minimal | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| Granuloma | Minimal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Microgranuloma | Minimal | 4 | 4 | 4 | 6 | 6 | 5 | 5 | 1 |
| | Mild | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Central hypertrophy of hepatocytes | Minimal | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 5 |
| | Mild | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Prostate | | | | | | | | | |
| Lymphocyte infiltration | Minimal | 3 | - | - | 5 | - | - | - | - |
| | Mild | 1 | - | - | 0 | - | - | - | - |
| Skeletal muscle | | | | | | | | | |
| Cell infiltration | Minimal | 1 | - | - | 0 | 0 | - | - | 0 |
| Spleen | | | | | | | | | |
| Increased hematopoiesis | Minimal | 1 | - | - | 1 | 0 | - | - | 0 |
| Glandular stomach | | | | | | | | | |
| Erosion | Minimal | 0 | - | - | 0 | 0 | - | 1[a)] | 1 |
| Thyroid | | | | | | | | | |
| Ectopic thymus | Minimal | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Interstitial cell infiltration | Minimal | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Remnant of ultimobranchial body | Minimal | 2 | 1 | 2 | 1 | 2 | 2 | 0 | 3 |
| Hypertrophy of follicular cells | Minimal | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 5 |
| Urinary bladder | | | | | | | | | |
| Submucosal cell infiltration | Minimal | 0 | - | - | 1 | 0 | - | - | 0 |

-, not examined

[a)] Number of examined animals was one in which dark red foci was grossly observed in the glandular stomach.

β-bromostyrene in corn oil through gavage at doses of 0, 30, 125, and 500 mg/kg/day for 28 days, and control and high dose groups were followed over a 14-day recovery period.

One female receiving 500 mg/kg was found dead on Day 3 of the dosing period. Decrease in spontaneous movement was observed in this rat on the first dosing day, but no clinical signs were observed thereafter. Gross and histopathological examinations revealed various changes in the dead rat; however, the cause of death was unclear. In surviving animals, decreases in spontaneous movements were observed only on the first dosing day, and salivation during handling was sporadically observed during Weeks 3 and 4 in both sexes in the 500 mg/kg group.

**Table 5.** Histopathological findings in the repeated dose 28-day oral toxicity study of β-bromostyrene in rats at the end of the recovery period.

| Organs / Findings | Sex | Males | | Females | |
|---|---|---|---|---|---|
| | Dose (mg/kg/day) | 0 | 500 | 0 | 500 |
| | Number | 6 | 6 | 6 | 5 |
| Kidney | | | | | |
| Tubular regeneration | Minimal | 3 | 4 | - | - |
| | Mild | 0 | 2 | - | - |
| Eosinophilic body in tubular cells | Minimal | 0 | 3 | - | - |
| Hyaline cast | Minimal | 0 | 1 | - | - |
| Interstitial mineralization | Minimal | 3 | 5 | - | - |
| Liver | | | | | |
| Periportal vacuolation of hepatocytes | Minimal | 1 | 0 | 1 | 2 |
| Microgranuloma | Minimal | 6 | 6 | 5 | 5 |
| Thyroid | | | | | |
| Ectopic thymus | Minimal | 1 | 0 | 0 | 0 |
| Remnant of ultimobranchial body | Minimal | 2 | 1 | 0 | 1 |
| Hypertrophy of follicular cells | Minimal | 0 | 1 | 0 | 0 |

-, not examined

Although food consumption in the 125 and 500 mg/kg groups were higher or lower than in the control group, toxicologically significant effects on body weight were not found throughout the study.

β-Bromostyrene clearly affected the kidneys in males; eosinophilic bodies in tubular cells, hyaline casts, and/or tubular degeneration were observed in the 125 and 500 mg/kg groups, and tubular regeneration was found after the recovery period. In the 500 mg/kg group, small round epithelial cells were found in the urine, which is indicative of renal tubular lesions. Increased urine volume and decreased urine osmolality are considered to be due to disturbances in tubular function. Interestingly, histopathological changes were observed only in males. Although slight but significant increases in urine volume and relative kidney weight were observed in females in the 500 mg/kg group, males were more susceptible to renal toxicity induced by β-bromostyrene. However, similar changes can be induced in females at higher doses.

In the present study, toxicological effects on the liver and thyroid were also observed. Hepatocyte hypertrophy was observed in both sexes in the 500 mg/kg group. Increased serum T-CHO, TG and PL levels indicate that β-bromostyrene affected lipid metabolism at 125 and 500 mg/kg in females. In the thyroid, follicular cell hypertrophy was found in the 125 and 500 mg/kg groups, which may be a compensatory response to increased hepatic metabolism of thyroid hormones.

In summary, β-bromostyrene affected the kidneys, liver, and thyroid of rats at doses of 125 mg/kg and above in our 28-day oral toxicity study. It was determined that the no-observed-adverse-effect-level of β-bromostyrene was 30 mg/kg/day for both sexes. Since all changes observed at the end of the dosing period disappeared or were reduced during the 14-day recovery period, toxic effects caused by β-bromostyrene are considered to be reversible.

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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