FINAL REPORT

Micronucleus Test of ETBE Using Bone Marrow of Rats of the “13-Week Toxicity Study of 2-Ethoxy-2-methylpropane in F344 rats (Drinking Water Study) [Preliminary Carcinogenicity Study]”

Study No.: 7046

June 29, 2007

<Translation from Japanese original into English>

Japan Industrial Safety and Health Association
Japan Bioassay Research Center
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Study Title
Micronucleus Test of ETBE Using Bone Marrow of Rats of the “13-Week Toxicity Study of 2-Ethoxy-2-methylpropane in F344 rats (Drinking Water Study) [Preliminary Carcinogenicity Study]”

Objective
The mutagenic potential of ETBE to induce micronuclei polychromatic erythrocytes was tested using the bone marrow of rats administrated ETBE in their drinking water for 13 weeks.

Guidelines
The present study was conducted following the “Guideline of micronucleus test on rodents” in the “Methods of Testing New Chemical Substances” of the Joint Notification of No.1121002 of the Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Heisei 15・11・13-No.2 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry and No.031121002 of the Environmental Policy Bureau, Ministry of the Environment, Japan, November 21, 2003.

The micronucleus test was conducted using bone marrow sampled from rats which were killed on schedule in the study entitled ”13-Week toxicity study of 2-ethoxy-2-methylpropane in F344 rats (drinking water study)“ [preliminary carcinogenicity study] (Study No. 0665).

GLP
The present study was performed in compliance with the good laboratory practice (GLP) Standards of the "The basis related to the examination institution which carried out an examination to modify new chemical substance" of the Joint Notification of No.1121003 of the Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Heisei 15・11・17-No.3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry and No.031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan, November 21, 2003 and also performed in compliance with "OECD Principles of Good Laboratory Practice" (November 26, 1997).

Sponsor
Japan Petroleum Energy Center Shin-Toranomon Building, 4-3-9, Toranomon, Minato-ku, Tokyo, Japan
Test Facility and facility manager
Japan Bioassay Research Center
Japan Industrial Safety and Health Association
2445 Hirasawa, Hadano, Kanagawa, Japan

Director: Shoji Fukushima, M.D., Ph. D.

Schedule of the Study
Commencement of the study: August 15, 2006
Receipt of animals: August 16, 2006
Allocation of animals: August 30, 2006
Initiation of test material treatment: August 30, 2006
Termination of test material treatment: November 30 and December 1, 2006
Scheduled necropsy and slide preparation: November 30 and December 1, 2006
Completion of the study: June 29, 2007

Study Personnel

Study director:
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Analysis of test substance, administration and management of test substance:
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Makoto Take (Analytical Chemistry Section, Department of Experimental Toxicology)

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Masaaki Suzuki (Animal Care Section, Department of Experimental Toxicology)
Toshiaki Sasaki (Animal Care Section, Department of Experimental Toxicology)
Tomoyuki Kamigaito (Animal Care Section, Department of Experimental Toxicology)
Kenji Takanobu (Animal Care Section, Department of Experimental Toxicology)
Archives of documents and samples

Protocol, specimens, raw data, test material, record documents, final report, certificate of quality assurance, the other documents and samples related to this study are to be stored in archives. Furthermore, 50 g of test compound are to be maintained in storage.

The retention period is for ten years (in principle) after submission of the final report. However, storage of specimens and test material will be restricted to the period for which their quality is maintained.

Signature, seal and date of the study director (person who prepared the final report)

Animal Care Section, Department of Experimental Toxicology

Study Director: <Signatured and sealed> <dated as June 29, 2007>
Tadashi Noguchi
Statement

Study title: Micronucleus Test of ETBE Using Bone Marrow of Rats of the “13-Week Toxicity Study of 2-Ethoxy-2-methylpropane in F344 rats (Drinking Water Study) [Preliminary Carcinogenicity Study]”

The present study was performed based on the protocol, and in compliance with the good laboratory practice (GLP) Standards of the "The basis which related to the examination institution which carried out an examination to modify new chemical substance" of the Joint Notification of No.1121003 of the Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Heisei 15・11・17-No.3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry and No.031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan, November 21, 2003 and also performed in compliance with "OECD Principles of Good Laboratory Practice" (November 26, 1997).

We consider the data generated by Japan Bioassay Research Center during the course of this study to be valid and that the final report fully and accurately reflects the raw data.

Japan Bioassay Research Center,
Japan Industrial Safety and Health Association.

Study director  <Signatured and sealed>  <Dated as June 29, 2007>
Tadashi Noguchi

Facility manager  <Signatured and sealed>  <Dated as June 29, 2007>
Shoji Fukushima, M.D., Ph.D.
Certificate of Quality Assurance

Study title: Micronucleus Test of ETBE Using Bone Marrow of Rats of the “13-Week Toxicity Study of 2-Ethoxy-2-methylpropane in F344 rats (Drinking Water Study) [Preliminary Carcinogenicity Study]”

Study No.: 7046

Test Substance: 2-Ethoxy-2-methylpropane (ETBE)

The study was conducted essentially in compliance with “The Standards Concerning Test Facilities Conducting the Tests Relating to New Chemical Substances” of the Joint Notification of No.1121003 of the Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Heisei 15・11・17-No.3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry and No.031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan, November 21, 2003 and "OECD Principles of Good Laboratory Practice" (November 26, 1997).

This report presents a full and accurate account of the results of the study.

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June 29, 2007

Quality Assurance Director

Attached to: Japan Bioassay Research Center
Japan Industrial Safety and Health Association

Title: Quality Assurance Manager

Signature: <Signatured> <Sealed>

Mitsuru Haresaku
Text

Micronucleus Test of ETBE Using Bone Marrow of Rats of the “13-Week Toxicity Study of 2-Ethoxy-2-methylpropane in F344 rats (Drinking Water Study) [Preliminary Carcinogenicity Study]”

Study No. 7046
Abstract

The mutagenic potential of 2-ethoxy-2-methylpropane (ETBE) to induce micronuclei (MN) was assessed by frequency of the micronucleated polychromatic erythrocytes (MNPCE) to polychromatic erythrocytes (PCE) in the bone marrow of rats.

The micronucleus test was conducted using rats killed on schedule in the study entitled “13-Week Toxicity Study of 2-Ethoxy-2-methylpropane in F344 rats (Drinking Water Study) [Preliminary Carcinogenicity Study]” (Study No. 0665). The rats of both sexes were given free access to drinking water containing the test chemical ETBE for 13 weeks. Animal husbandry and administration of the test chemical were appropriately managed.

Although there was no mortality during the treatment period of 13 weeks, retardation of body weight gain was noted in the males treated with 10000 ppm ETBE.

The micronucleus test was conducted for both sexes of the control and three treated (1600, 4000 and 10000 ppm ETBE) groups. Each group consisted of 10 rats of either sex. At sacrifice, bone marrow cells were collected from the femur, smeared on slides and then the frequencies of micronucleated PCE in PCE (MNPCE/PCE) and the ratios of PCE to the total number of erythrocytes (PCE/E) were calculated.

The ratios of MNPCE/PCE in males were 0.16±0.08% in the control group, 0.17±0.12% in the 1600 ppm ETBE group, 0.14±0.06% in the 4000 ppm ETBE group, and 0.19±0.15% in the 10000 ppm ETBE group. Those in females were 0.10±0.07% in the control group, 0.16±0.09% in the 1600 ppm ETBE group, 0.09±0.06% in the 4000 ppm ETBE group, and 0.13±0.07% in the 10000 ppm ETBE group. As the values of the controls were within the background data of F344 rats in our facility, it was evident to this study was appropriately conducted. No statistical significant differences in the ratios (MNPCE/PCE) of MNPCE to PCE were observed in either sex of the treated groups, as compared to the control values. In addition, no tendencies for dose-dependent increase in the MNPCE/PCE were found in either sex of the treated groups. No alterations of the PCE/E ratio were also noted.

It was considered that the highest level of ETBE was appropriately selected for this study, since retardation of body weight gain was observed in males receiving 10000 ppm ETBE.

Thus, from these results, ETBE was assessed to be negative on micronucleus induction for bone marrow in rats administered ETBE (drinking water) for 13 weeks.
I  Test material
I-1  Specifications of the test material
I-1-1  Name of the test material
   Name: 2-Ethoxy-2-methylpropane
   Synonym: Ethyl tertiary-butyl ether (ETBE)
   CAS No.: 637-92-3

I-1-2  Chemical structure and molecular weight
   Chemical formula:
   \[
   \begin{align*}
   &\text{CH}_3 \\
   &\text{H}_3\text{C} - \text{C} - \text{O} - \text{CH}_2 - \text{CH}_3 \\
   &\text{CH}_3
   \end{align*}
   \]
   Molecular weight: 102.17

I-1-3  Physicochemical properties of the test material
   Appearance: Colorless transparent liquid.
   Boiling point: 70°C
   Vapor pressure: 17 kPa (25°C)
   Specific gravity: 0.74 (25°C/4°C)
   Solubility: Slightly soluble in water 1.2g/100g, 20°C
   Storage condition: At room temperature in a dark place.

I-2  Test material used in this bioassay
   Supplier: Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan)
   Grade: Grade 1 (Highly purified product) of Tokyo Chemical Industry Co., Ltd.
   Purity: 99.3% (Report from the supplier)
   Lot No.: R74EE
I-3 Specificity and identity, and stability of the test material

I-3-1 Analytical method for specificity and identity

The test material was analyzed by electron impact mass spectrometry (Agilent Technologies 5973N) and its identity was confirmed by comparison with reported values.

As a result, a mass spectrum of test material showed a fragment peak the same as the reported value (Reference 1), thus confirming the compound to be 2-ethoxy-2-methylpropane (Reference 2).

I-3-2 Stability of the test material

The stability of the test material was assessed by gas chromatography (Hewlett Packard 5890A) at before the beginning and at the end of use of the test material, and confirmed by comparison of the findings.

As a result, there was no difference between the results of the beginning and the end of use of the test material, and stability during the treatment period was therefore confirmed (Reference 2).

I-4 Animals

Both sexes of F344/DuCrClCrj (Fischer) rats (SPF animals, received from Charles River Laboratories Japan Inc. (795 Shimo-furusawa, Atsugi, Kanagawa, Japan) ) for use in the carcinogenicity study of ETBE, were employed in this study.

Totals of 75 animals of each sex were obtained at four weeks of age and allowed one week of quarantine and a one week acclimation period, and then 60 males and 60 females (body weight ranges: males, 116-131g; females, 94-103g) were selected for the study. These animals showed no abnormality in general condition and body weight gain, and were near to the median body weight value.

F344/DuCrClCrj rats (SPF animals) were selected because of their genetic stability, their routine use for carcinogenicity studies, their low spontaneous carcinogenicity, and their high sensitivity to carcinogenicity of chemicals.
II  Study methods
II-1  Administration

II-1-1  Administration route

Oral treatment of the test material was selected as the administration route for this study.

II-1-2  Methods of administration of the test material

The test material was dissolved in drinking water at the test doses and given to rats using a pressurized type water supply tank under air pressure (0.05 MPa). The tank was connected to the automatic water supply available to the animals. The pressurizing type water supply tank with fresh water containing the test material was exchanged once a week.

II-1-3  Study period

The duration of administration by drinking water was for 13 weeks, this treatment being continued until immediately before necropsy for each animal.

II-1-4  Administration concentration

The administration concentrations were set at three levels of 1600, 4000 and 10000 ppm (weight ratio w/w) with a common ratio of 2.5. In addition, animals in the control group received the vehicle, deionized water [Hadano City Water was filtered, UV-irradiated, deionized, and then filtered again].

II-1-5  The reason for the selection of the route and method of test material administration

Oral administration by drinking water was selected as the administration route, since the test material is soluble in water, and in line with the possible human exposure route of ETBE. The duration of administration was decided to 13 weeks in order to determine doses for a long-term carcinogenicity test.

The doses in this micronucleus test were determined as a result of conferring with the sponsor, based on the results of a 2-week range finding study (Study no. 4410; Japan Bioassay Research Center) and a previous 28-day repeated dose oral toxicity study in rats (Japan Petroleum Energy Center, Ref. 3).

In the 2-week range finding study, both sexes of F344/DuCrIcrj (Fischer) rat (SPF animals) were given free access to drinking water containing 1000, 5000 or 10000 ppm (w/w) ETBE, and general condition, body weight, food and water consumptions were investigated. No mortality was found in either sex of any group. The body weights and food consumption in rats given 5000 ppm and 10000 ppm ETBE were comparable to the control values after day 3. Water consumption in rats given 1000 ppm, 5000 ppm and 10000 ppm ETBE was slightly lower than in the control group during the course of the study. Final body weights at the end of the treatment in male 1000, 5000 and 10000 ppm groups were 100%, 101% and 94%, respectively, and those in female 1000, 5000 and 10000 ppm groups were 101%, 100% and 100%, respectively, of the control values. Average test material intake in male 1000, 5000 and 10000 ppm groups was 102 mg/kg/day, 441 mg/kg/day and 807 mg/kg/day, respectively, and in female 1000, 5000 and 10000 ppm groups was 103 mg/kg/day, 470 mg/kg/day and 1000 mg/kg/day, respectively, generally consistent with the concentration of test material in the drinking water.

In the 28-day repeated dose oral toxicity study, both sexes of 5-week old Sprague-Dawley rats were administered ETBE by gavage at doses of 15, 25, 50, 100, 150, 400 and 1000 mg/kg/day. As results, altered general conditions such as decreased activity level were observed in rats given more than 100
mg/kg/day, increased liver weights were found in rats given 400 and 1000 mg/kg/day, and histopathological alterations of the liver were noted in rats given 1000 mg/kg/day. From these effects, it was reported that no-observed-effect level (NOEL) was 50 mg/kg/day, and the no-observed-adverse-effect level (NOAEL) was 150 mg/kg/day.

From the aforementioned results, it was considered that the highest dose level of ETBE used in this study should be 10000 ppm, which induced the increase in liver weight and histopathological alterations in the liver. As solubility in water of 2-ethoxy-2-methylpropane is 1.2 g/100g (12,000 ppm), a 10,000 ppm concentration of ETBE in the drinking water is close to the maximum technically feasible dose in drinking water. The lowest dose level of ETBE in drinking water was estimated to be 500 ppm, which corresponded to the NOEL of 50 mg/kg/day in the 28-day toxicity study of ETBE. However, taking the treatment period of 13 weeks into consideration, 250 ppm (half of the NOEL) was selected as appropriate for the lowest dose in this study.

Therefore, the highest dose level was selected as 10000 ppm (w/w) for the 13-week toxicity study, and lower levels were selected as 4000, 1600, 640 and 250 ppm with a common ratio of 2.5.

II-1-6 Preparation of test material in drinking water

Deionized water was poured into a water tank, weighed, and then weighed test material was added. After covering with the lid and sealing, ETBE was dissolved by rotating the water tank using the ball mill pot rotary for one hour, under increased air pressure (0.05 MPa).

The frequency of preparation of dosing water (drinking water) was once a week, at which times the pressurized water tank was replaced. ETBE concentrations in the present study are indicated as ppm (w/w).

II-1-7 Concentration of the test material in drinking water at the first preparation

Test material concentrations in drinking water at each level were analyzed at the first preparation. ETBE concentrations in drinking water in the pressurized water tank were determined at sampling 3 points each for each level by gas chromatography (Hewlett Packard 5890A) with a head-spaced sampler (Hewlett Packard 7694), and confirmed to be within the acceptable ranges.
Analytical results for achieved concentrations in each group were 91.2% - 110% of the intended values. Thus, it was confirmed that test material concentrations in drinking water containing test material were acceptably close to the intended concentrations.

II-1-8 Stability of the test material in drinking water

Stability of the test material in drinking water containing the test material at the lowest dose of 250 and the highest dose of 10,000 ppm was analyzed in the 13-week oral toxicity study (preliminary study for the carcinogenicity study)(Study no. 0665). Drinking water containing 250 or 10,000 ppm ETBE in pressurized water tanks (0.05 MPa), was stored in an animal room at room temperature for 8 days, and then analyzed by gas chromatography (Hewlett Packard 5890A) with a head-spaced sampler (Hewlett Packard 7694).

Analytical results for achieved concentrations in the 250 ppm and 10,000 ppm groups after 8 days storage were 94% and 108%, respectively, of the initial values. Thus, it was confirmed that test material in drinking water at both concentrations was stable for the period without replacement (Ref. 2).

II-2 Animal management

II-2-1 Number of animals of each group

Three higher-dose treatment groups in the study entitled “13-Week Toxicity Study of 2-Ethoxy-2-methylpropane in F344 rats (Drinking Water Study) [Preliminary Carcinogenicity Study]” and one control group, for a total of 4 groups (10 rats/sex/group) were employed in the present study.

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<thead>
<tr>
<th>Group name</th>
<th>Male</th>
<th>Female</th>
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<td>Control group</td>
<td>10 (1001-1010)</td>
<td>10 (2001-2010)</td>
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<td>1600 ppm group</td>
<td>10 (1301-1310)</td>
<td>10 (2301-2310)</td>
</tr>
<tr>
<td>4000 ppm group</td>
<td>10 (1401-1410)</td>
<td>10 (2401-2410)</td>
</tr>
<tr>
<td>10000 ppm group</td>
<td>10 (1501-1510)</td>
<td>10 (2501-2510)</td>
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II-2-2 Allocation and individual identification methods

Allocation was carried out by a method to minimize the deviation of body weights among groups (proper stratification method) (Reference 4). First, animals which were normal for general condition and body weight gain, were allotted in order of heaviest body weight. Second, comparing with the total weight of each group, heaviest weight animals were, in turn, allotted to the lightest weight groups, this procedure then being repeated.

During the quarantine/acclimation period, individual animals were identified by tail marking with a felt-pen. During the treatment period, individual animals were identified by ear punch. In addition, a label carrying individual animal numbers was affixed to each cage.

The animals were housed in independent rooms in a barrier area, and the study number, animal species and animal numbers were displayed on the doors of the rooms to allow distinction from other studies and species of animals.

II-2-3 Animal husbandry

(1) Housing conditions

Animals were housed in an animal room (room no: 606) during the quarantine/acclimation period, and in another animal room (room no: 603) during the administration period.

Environmental conditions of animal rooms are shown below. The actual values (mean ± standard deviation) for temperature and relative humidity of animal room are given in “< >”. No changes that could have affected conditions of health of the animals and micronucleus inductions in bone marrow cells were apparent in the environment for either animal room (Reference 2).  

Temperature: 23±2°C
<Room No. 606: 23.1±0.1°C (23.0-23.2°C)>  
<Room No. 603: 23.0±0.5°C (22.3-24.5°C)>

Relative humidity: 55±15%
<Room No. 606: 54±2%>
<Room No. 603: 54±3%>

Lighting: Artificially illuminated for 12h (8:00 to 20:00) and 12h off (20:00 to 8:00)
Ventilation: 15-17 times/hr
Accommodation method: One animal to a cage  
Materials/shape/dimensions of cages: Two-linked stainless steel cages [170 (W) x 294 (D) x 176 (H) mm/animal]

(2) Food

Rats were given ad libitum access to CRF-1 pellet diet (sterilized by 30 kGy-γ rays irradiation) obtained from Oriental Yeast Co., Ltd. (Chiba factory: 8-2 Shinminato, Mihama-ku, Chiba, Japan) placed in feeders for pellets throughout the experimental period. However, the animals were fasted from the evening of the previous day for scheduled necropsy.

Analytical data for nutrients for each lot used in the present study were obtained from Oriental Yeast Co., Ltd., and archived. Analytical data for contaminants in diet for each lot used in the present study were also received from Japan Food Research Laboratories, Foundation (52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo, Japan), confirmed to be within acceptable ranges (see protocol), and then archived.

Study No.7046
(3) Drinking water

In the quarantine and acclimation periods, animals received filtered and ultraviolet irradiated tap water (Hadano-shi, Kanagawa water service station supply) ad libitum, via automatic water suppliers, as their drinking water. In the treatment period, treated animals were similarly given ad libitum access, via automatic water suppliers, to drinking water containing the test material at the targeted concentrations prepared with deionized water, and control animals received deionized water as the drinking water.

Drinking water was routinely sampled, and shipped to Food and Drug Safety Center, Hatano Research Institute, Japan (729-5 Ochiai, Hadano, Kanagawa, Japan) for analyses, performed in accordance with Water Supply Act. Analytical results were received, and contaminant levels were confirmed to be within acceptable ranges, and then archived.

II-3 Observation and laboratory investigations, and methods

II-3-1 Observation of status and general conditions

Animals were observed for their status (alive, dead and in extremis) daily, and also for detailed general condition once a week.

No treatment related mortalities and changes in general condition were found in any group.

II-3-2 Measurement of body weights

Animals were weighed weekly for all 13 weeks.

Retardation of body weight gain was noted in males of the 10000 ppm ETBE group throughout the treatment period, and also in the female 10000 ppm ETBE group in the early phase (until week 2) of the study (Ref. 2).

II-4 Micronucleus test

The micronucleus test was conducted using rats which were killed on the scheduled date in a study entitled “13-Week toxicity study of 2-ethoxy-2-methylpropane in F344 rats (Drinking water study) [Preliminary carcinogenicity study]” (Study No. 0665). Femurs were excised from rats of the control group and three treated groups (1600, 4000 and 10000 ETBE groups). Bone marrow cells were collected from the femurs, smeared on slides, stained with fluorescent Acridine Orange, and then observed microscopically.
II-4-1  Preparation of specimens and microscopic observation (Ref. 5)

(1) Preparation of specimens

Bone marrow cells were harvested from the left femur by flushing of 1 mL fetal bovine serum with a syringe into a centrifuge tube (10 mL). Cell suspensions were centrifuged at 1000 rpm (centrifuge KS5200 and rotor ST-480, Kubota Co., Ltd., Japan) for 5 minutes, and the supernatant was discarded, to allow marrow cell suspensions to be made by adding a small amount of fetal bovine serum. A small drop of suspension was placed on a slide, and a smear was prepared (2 slides/animal). Slides were air dried at room temperature and fixed with methanol for 5 minutes. Microscopic observation was performed just after fluorescent staining with Acridine Orange. Acridine Orange staining was accomplished by dropping 20 μL of Acridine Orange solution (40 μg/mL) on the smear, and mounted under a cover glass.

(2) Microscopic observation of specimens

1) Microscopic observation

Just after staining, bone marrow smears were microscopically observed in two areas, demonstrating well-smeared conditions, on each slide. One thousand polychromic erythrocytes (PCE) in each area were counted and the number of micronucleated polychromatric erythrocytes (MNPCE) in PCE was determined, to calculate the ratio (MNPCE/PCE). In addition, five hundred erythrocytes (polychromatic and normochromatic) were examined, and the number of polychromatric erythrocytes was counted, and the ratio (PCE/E) calculated.

Observation was carried out by a blinded method using high magnification (x 400) with a fluorescent microscope (BX51, Olympus Co., Ltd., Japan)

2) Distinction of polychromatric erythrocytes from normochromatric erythrocytes

Anucleate cells emitting red fluorescence were counted as polychromatric erythrocytes and those did not were diagnosed as normochromatric erythrocytes.

3) Identification (discrimination) of micronuclei

Size: The upper limit was half of a diameter of an normochromatric erythrocyte, and the lower limit was the size that could be distinguished.

Shape: The circle is the main form, but the following forms are also found: oval, crescent, doughnut, and hemicycle etc.

Color: Should be same as the nuclei of adjacent nucleated cells.

Outline: Should be clear because micronucleus has a nuclear membrane.

Focus: Granules suggestive of micronuclei in the cells were confirmed by moving the focus up and down.
II-5 Numerical value processing and statistical methods

II-5-1 Handling of numerical values, and their display

All numerical data are presented depending on the precision of the measuring apparatus.

Body weights (grams as the unit) were measured to 1 digit of integer value and shown as such.

The frequency (% as the unit) of micronucleated polychromatic erythrocytes (MNPCE) was rounded off to three decimal places, and shown as two decimal places.

The ratio (PCE/E) (% as the unit) of the number of polychromatic erythrocytes (PCE) to the total number of erythrocytes (E) was rounded off to two decimal places, and shown as one decimal place.

II-5-2 Statistical processing

Statistical comparison between control and treated groups of numerical data obtained for body weights was conducted using the Bartlett’s test. If homogenous, the data were analyzed with respect to one-way layout variance, and if significant differences were found the Dunnett’s multiple comparison test was applied. If not homogenous, the data were ranked through each group, and analyzed by Kruskal-Wallis’s test, followed by a multiple comparison test of Dunnett’s type when a significant difference was found between groups.

For the frequency of micronucleated PCE (MNPCE/PCE), the data was determined by the method of Kastenbaum and Bowman at a significance level 1% (Re. 6). The ratio (PCE/E) of the number of polychromatic erythrocytes (PCE) to the total number of erythrocytes (E) was analyzed with the Wilcoxon rank sum test at a significance level 5% (Ref. 7).

III. Results and discussion

The micronucleus test was conducted using rats killed on schedule in the study entitled “13-Week Toxicity Study of 2-Ethoxy-2-methylpropane in F344 rats (Drinking Water Study) [Preliminary Carcinogenicity Study]” (Study No. 0665). The rats of both sexes were given free access to drinking water containing the test chemical ETBE for 13 weeks. Animal husbandry and administration of the test chemical were appropriately managed.

Although no mortality was found during the treatment period of 13 weeks, retardation of body weight gain was noted in the males treated with 10000 ppm ETBE.

For reference, the test material intake in the treatment groups was as follows: the 1600 ppm ETBE group, \(101\pm21\) mg/kg /day for males and \(120\pm22\) mg/kg /day for females; the 4000 ppm ETBE group, \(259\pm41\) mg/kg /day for males and \(267\pm50\) mg/kg /day for females; and the 10000 ppm ETBE group, \(626\pm91\) mg/kg /day for males and \(629\pm114\) mg/kg/day for females (Ref. 2).

The micronucleus test was conducted for the control and three treated groups receiving 1600, 4000 and 10000 ppm ETBE in drinking water for 13 weeks. Each group consisted of 10 rats of either sex. At sacrifice, the bone marrow cells were collected from the femur, smears were prepared, and then the frequency (MNPCE/PCE) of micronucleated polychromatic erythrocytes (MNPCE) and the ratio (PCE/E) of polychromatic erythrocytes (PCE) to the total number of erythrocytes (E) were calculated.

The results of the micronucleus test are shown in Tables 1 and 2 and Figure 1.

The frequencies of MNPCE/PCE in males were 0.16±0.08% in the control group, 0.17±0.12% in the 1600 ppm ETBE group, 0.14±0.06% in the 4000 ppm ETBE group, and 0.19±0.15% in the 10000 ppm ETBE group. Those in females were 0.10±0.07% in the control group, 0.16±0.09% in the 1600 ppm
ETBE group, 0.09±0.06% in the 4000 ppm ETBE group, and 0.13±0.07% in the 10000 ppm ETBE group. No statistical significant increases as compared to control values, or dose-dependent tendencies for increase, in the frequencies of MNPCE/PCE were noted in either sex of the treated groups. In addition, no significant changes in the PCE/E ratio were found in either sex of the treated groups.

IV Judgment of the results

It was considered that the highest level of ETBE was appropriately selected for this study, since retardation of body weight gain was observed in males receiving 10000 ppm ETBE.

As the frequencies of MNPCE/PCE for the controls were within the background data (0.15±0.09%, N=97) for F344 rats in our facility, it was evident that this study was appropriately conducted. No statistical significant differences in the ratios (MNPCE/PCE) of MNPCE to PCE were observed in either sex of the treated groups, as compared to the control values. In addition, no dose-dependent tendencies for increase of MNPCE/PCE were found in either sex of treated groups.

Thus, from these results, ETBE was assessed to be negative on micronucleus induction for bone marrow in rats administered ETBE (in drinking water) for 13 weeks.

V Unforeseeable circumstances that might have affected the reliability of the study and deviations from the protocol

No particulars require mention, which might have affected the reliability of the study. In addition, no particulars require mention of deviations from the protocol.

References


### Table 1: Results of micronucleus test (Male) Drinking water study for 13 weeks

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<th>GROUP NAME</th>
<th>ANIMAL ID NO.</th>
<th>SLIDE ID NO.</th>
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<th>MNPCE/10000PCE (%)</th>
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<td>23.8 ± 5.4</td>
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</table>

a) Observation region on the slide.

b) Minimum value of MNPCE/PCE (%) in each group.

c) Maximum value of MNPCE/PCE (%) in each group.

d) Total number of MNPCE observed in each group.

e) Average±SD of PCE/E (%) in each group.

f) Average±SD of MNPCE/PCE (%) in each group.

PCE : Polychromatic erythrocytes

E : Erythrocytes (Polychromatic and Normochromatic erythrocytes)

MNPCE : Micronucleated polychromatic erythrocytes
Table 1 Results of micronucleus test (Female) -Continued-

Drinking water study for 13 weeks

Test substance : 2-Ethoxy-2-methylpropane

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<th>GROUP NAME</th>
<th>ANIMAL ID NO</th>
<th>SLIDE ID NO</th>
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a) Observation region on the slide.
b) Minimum value of MNPCE/PCE (%) in each group.
c) Maximum value of MNPCE/PCE (%) in each group.
d) Total number of MNPCE observed 10000PCE in each group.
e) Average±SD of PCE/E (%) in each group.
f) Average±SD of MNPCE/PCE (%) in each group.

PCE : Polychromatic erythrocytes
E : Erythrocytes (Polychromatic and Normochromatic erythrocytes)
MNPCE : Micronucleated polychromatic erythrocytes
Table 2  Results of micronucleus test (Male)
Drinking water study for 13 weeks

<table>
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<tr>
<th>GROUP NAME</th>
<th>NUMBER OF ANIMALS</th>
<th>NUMBER OF OBSERVED CELLS (Cells/Animal)</th>
<th>Ratio of PCE/E (%)</th>
<th>Frequencies of MNPCE/PCE (%)</th>
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</thead>
<tbody>
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<td>Control Group</td>
<td>10</td>
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<td>24.2 ± 3.5&lt;sup&gt;a&lt;/sup&gt; (18.1&lt;sup&gt;b&lt;/sup&gt; - 29.4&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>0.16 ± 0.08&lt;sup&gt;d&lt;/sup&gt; (0.05&lt;sup&gt;e&lt;/sup&gt; - 0.30&lt;sup&gt;f&lt;/sup&gt;, 31&lt;sup&gt;g&lt;/sup&gt;)</td>
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<td>1600 ppm Group</td>
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<td>26.3 ± 5.4 (17.3 - 35.3)</td>
<td>0.17 ± 0.12 (0 - 0.35, 34)</td>
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<td>24.7 ± 4.9 (17.7 - 32.3)</td>
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<td>10000 ppm Group</td>
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<td>23.8 ± 5.4 (16.5 - 31.4)</td>
<td>0.19 ± 0.15 (0 - 0.45, 38)</td>
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<sup>a</sup>) Average±SD of PCE/E(%)
<sup>b</sup>) Minimum value of PCE/E(%)
<sup>c</sup>) Maximum value of PCE/E(%)
<sup>d</sup>) Average±SD of MNPCE/PCE(%)
<sup>e</sup>) Minimum value of MNPCE/PCE(%)
<sup>f</sup>) Maximum value of MNPCE/PCE(%)
<sup>g</sup>) Total number of MNPCE observed 20000PCE in each group

PCE : Polychromatic erythrocytes
E : Erythrocytes (Polychromatic and Normochromatic erythrocytes)
MNPCE : Micronucleated polychromatic erythrocytes
### Table 2  Results of micronucleus test (Female)  -Continued-

Drinking water study for 13 weeks

Test substance : 2-Ethoxy-2-methylpropane

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<th>Ratio of PCE/E (%)</th>
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<td>23.7±3.8(^a) (18.1(^b) - 29.4(^c))</td>
<td>0.10±0.07(^d) (0.05(^e) - 0.25(^f), 19 (^g))</td>
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<td>1600 ppm Group</td>
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<td>24.7±3.4 (21.1 - 30.3 )</td>
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<td>24.8±2.6 (20.5 - 29.8 )</td>
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<td>23.6±4.2 (15.1 - 29.3 )</td>
<td>0.13±0.07 (0.05 - 0.25, 25 )</td>
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- \(^a\) Average±SD of PCE/E(%)
- \(^b\) Minimum value of PCE/E(%)
- \(^c\) Maximum value of PCE/E(%)
- \(^d\) Average±SD of MNPCE/PCE(%)
- \(^e\) Minimum value of MNPCE/PCE(%)
- \(^f\) Maximum value of MNPCE/PCE(%)
- \(^g\) Total number of MNPCE observed 20000PCE in each group

**PCE** : Polychromatic erythrocytes
**E** : Erythrocytes (Polychromatic and Normochromatic erythrocytes)
**MNPCE** : Micronucleated polychromatic erythrocytes
Fig. 1 Results of Micronucleus test
Drinking water study for 13 weeks

Test substance: 2-Ethoxy-2-methylpropane