3. Group size: $n_s = 6, n_m = 6, n = 42$.
4. Mean $\pm$ standard deviation of observed values

$$(d_A, d_B) = (0, 0) : 91.2 \pm 16.2$$
$$(d_A, d_B) = (0.1, 0) : 92.3 \pm 9.7, \quad (d_A, d_B) = (0.2, 0) : 96.5 \pm 5.4$$
$$(d_A, d_B) = (0, 12.5) : 165.4 \pm 27.0, \quad (d_A, d_B) = (0, 25) : 220.8 \pm 27.5$$
$$(d_A, d_B) = (0.05, 6.25) : 141.8 \pm 12.8, \quad (d_A, d_B) = (0.1, 12.5) : 183.5 \pm 10.3$$

5. Estimated value of Δ: 19.5.
6. Result of t-test: significant in one-sided Welch test with significance level 2.5% $T = 3.98$, $\nu = 31$, $p < 0.01$.

From the above results, we suggested that the combinations for these two agents were synergistic. The synergism was observed in the real situations such as endocrine disruptor study.

4.2. Recommended group point selection

The example introduced in the preceding section has two simultaneous administration groups. In this section, we investigate the most appropriate dose level among several dose levels in the case of one simultaneous administration group. The conditions in the investigation are as follows. The conditions are set pursuant to the example in the preceding section except for assuming $\sigma = 1$ without losing any generality.

4.2.1. Fixed condition.

1. Group points for single administration: $(d_A, d_B) = (0, 0), (1, 0), (2, 0), (0, 1), (0, 2)$.
2. Group size: $n_s = 6, n_m = 12$.
3. Parameters in the dose–response curve: $\beta_0 = 1.0, \beta_A = 1.0, \beta_B = 1.0$.
4. Variance σ^2: $\sigma_s^2 = \sigma_m^2 = 1.0$.
5. Nominal significance level of t-test: one-sided 2.5%.

4.2.2. Varied condition.

6. Group points for simultaneous administration: $d_A = 0.1(0.1)2.0$, $d_B = 0.1(0.1)2.0$.
7. Strength of synergism Δ:
   Case 1 (constant case): $\Delta = 1.0$
   Case 2 (square root case): $\Delta = 0.8\sqrt{(d_A + d_B)}$
   Case 3 (linear case): $\Delta = 0.6(d_A + d_B)$

Numerical calculations were performed to calculate the power under the above conditions. The left-hand side of Figures 1–3 shows a three-dimensional display on the vertical axis above the dose plane of the power in Cases 1–3, respectively. On the other hand, the right-hand side of Figures 1–3 represents power functions when $d_A = d_B$ at the dose level for simultaneous administration group.

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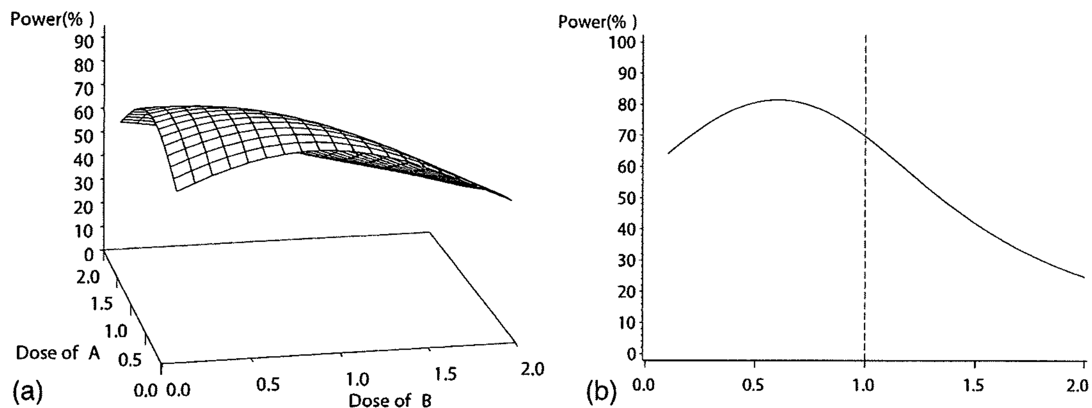

Figure 1. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a constant surplus case (Case 1)

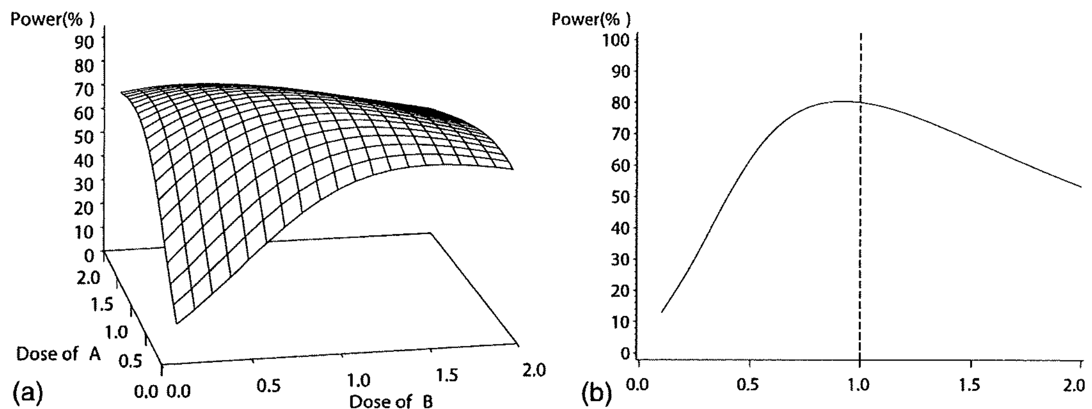

Figure 2. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a square root surplus case (Case 2)

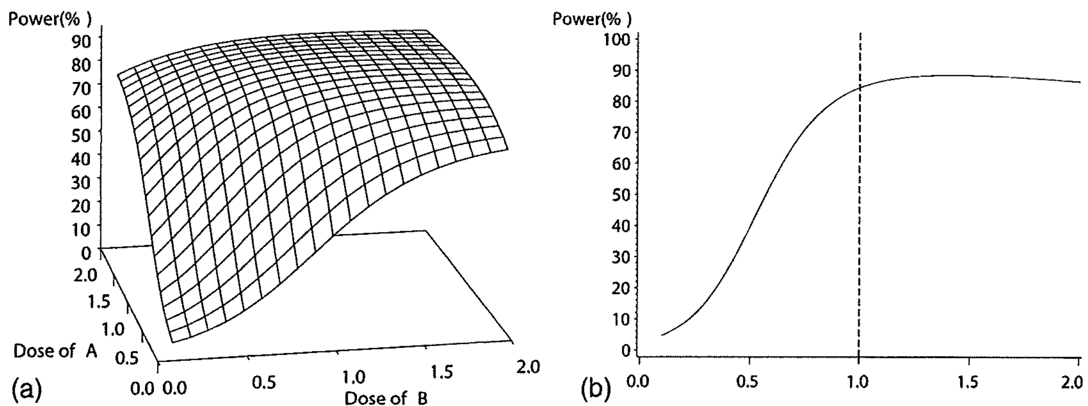

Figure 3. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a linear surplus case (Case 3)

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According to Figure 1, the power is high when the group point of the simultaneous administration group is within the triangular region and the power is low when it is located outside that region. Consequently, the design to evaluate synergism should have the group point within the triangle region. Figure 2 shows the power reaches a peak near the boundary of the triangular region and decreases apart from the triangular region as the dose increases. In consequence, the group point should be located on the boundary of triangle region. Finally, according to Figure 3, the power achieves the steady state on the boundary of the triangular region and reaches a peak slightly outside the triangular region when Δ becomes larger linearly as the dose increases. In numerical example, recommended group point in Cases 1–3 is, respectively, $(d_A, d_B) = (0.6, 0.6), (0.9, 0.9), (1.4, 1.4)$ and the power is 81.4%, 80.4%, 88.4%, respectively.

The sensitivity analysis was conducted to investigate the usefulness of location of the group point in the real study, such as two group points $(d_A, d_B) = (0.5, 0.5)$ and $(1.0, 1.0)$. In this section, only group points for simultaneous administration were changed in the following way and all other conditions were the same.

4.2.3. Group points for simultaneous administration.

(1) $(d_A, d_B) = (0.6, 0.6)$ (recommended in Case 1)
(2) $(d_A, d_B) = (0.9, 0.9)$ (recommended in Case 2)
(3) $(d_A, d_B) = (1.4, 1.4)$ (recommended in Case 3)
(4) $(d_A, d_B) = (1.0, 1.0)$ (on the boundary)
(5) $(d_A, d_B) = (0.5, 0.5), (1.0, 1.0)$ (real study)

Here, the total sample size for simultaneous administration groups is fixed in all group points (1)–(5). Then n_m for two groups' simultaneous design such as (5) is half of that for one group's design.

The numerical calculation was conducted under the above conditions. Table 2 summarizes the reduction of power from a recommended group point. When the group point is located close to the boundary of the triangular region, the reductions of power from a recommended group point are small. Furthermore, in the situation of real studies, the loss of power is negligible compared to that in a recommended group point. It is considered that the configuration of the group points in real study is reasonable from the statistical viewpoint.

4.3. Recommended group size

In this section, under conditions in which the total number of animals is fixed and the effect sizes are varied, we investigate the group size of the simultaneous administration group that maximizes the power.

Table 2. Reduction of power from a recommended group point in three cases

Group point (d_A, d_B)	Case 1 (constant case)	Case 2 (square root case)	Case 3 (linear case)
(0.6, 0.6)	0.0	−10.2	−34.7
(0.9, 0,9)	−7.3	0.0	−9.3
(1.4, 1.4)	−34.6	−9.7	0.0
(1.0, 1.0)	−12.2	−0.4	−5.1
(0.5, 0.5), (1.0, 1.0)	−2.0	−2.6	−19.7

n_m for two group points is half of that for one group.

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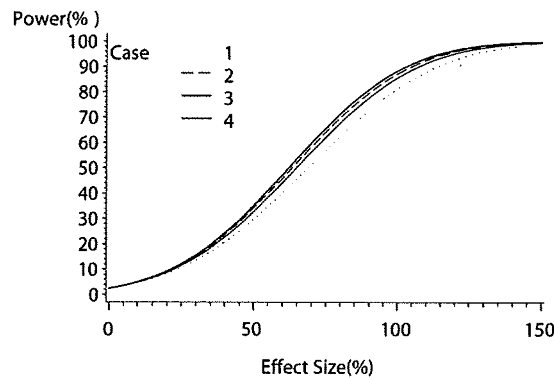

Figure 4. Relation between power and group size in four cases

4.3.1. Fixed conditions.

1. Group points for single administration: $(d_A, d_B) = (0, 0), (1, 0), (2, 0), (0, 1), (0, 2)$.
2. Group point for simultaneous administration: $(d_A, d_B) = (0.6, 0.6)$.
3. Parameters in the dose–response curve: $\beta_0 = 1.0, \beta_A = 1.0, \beta_B = 1.0$.
4. Variance σ^2: $\sigma_s^2 = \sigma_m^2 = 1.0$.
5. Nominal significance level of t-test: one-sided 2.5%.
6. Total number of animals: $n = 42$.

4.3.2. Varied conditions.

7. Group size:
   Case 1: $n_s = 6, n_m = 12$
   Case 2: $n_s = 5, n_m = 17$
   Case 3: $n_s = 4, n_m = 22$
   Case 4: $n_s = 3, n_m = 27$
8. Effect Size: $\Delta/\sigma_m = 0.1(0.1)1.5$.

Figure 4 shows the results of the numerical calculations, with the power on the vertical axis and the effect size on the horizontal axis. According to Figure 4, regardless of the effect size, Case 3 gives the maximum power. Because the group size is constrained to be an integer, it is not possible to give the most appropriate group size as a continuous value. The power becomes larger when the group size of the simultaneous administration group is set larger than that of the single administration group. When $n = 42$, what is prominent is the fact that the group size of 22 in the simultaneous administration group is nearly equal to the total number of animals in the single administration group, $4 \times 5 = 20$.

5. DISCUSSION

5.1. Conclusion under assumed conditions

In the previous section, we investigated applicable designs assuming that the response variables follow a homoscedastic normal distribution, that the dose–response relationship for single administration is

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linear, that synergism is defined as a larger response obtained with simultaneous administration as compared to the dose plane obtained with single administration by assuming additivity, that there are five groups for single administration and one group for simultaneous administration, etc.

As the results of numerical calculation for the group point, when the departure Δ from additivity is proportional to square root of the dose, it is revealed that the group point for the simultaneous administration group should be on the boundary of the triangle region. Here, the results seemed to be reasonable. Because it is natural to expect that the departure Δ does not continue to increase along with the dose constantly. So, we applied these two situations as the sensitivity analysis. When the departure Δ become larger linearly along with the dose, the group point should be located slightly outside the triangle region. On the other hand, the group point should be set inside the triangle region when the departure Δ is constant. However, the group point should be placed on the boundary of the triangular region, because the reduction of power from a recommended group point is small. This means that the conventional design in the real study is appropriate.

Subsequently, with respect to the group size, we revealed that the total number of animals allocated to the simultaneous administration group should be same size as that in the single administration group.

5.2. Heteroscedasticity

When heteroscedasticity in data is expected from the past research, it is required to adjust the degree of freedom by using the Welch test. For the cases discussed in this paper, we used the Welch test, which is robust for heteroscedasticity because there is a tendency in real data for the variance to increase with an increase in responses although the number of animals is small. However, the Welch test does not control a type 1 error below the nominal significance level under heteroscedasticity. It is, therefore, required to confirm, *ex post facto*, the type 1 error when the degree of heteroscedasticity is large.

5.3. Linearity

For the cases discussed in this paper, based on advanced information, it was possible to select the dose so that there is linearity with the single administration group. This was easier due to the fact that the number of dose levels in the single administration group was small (three levels). From the experimental results, it was also confirmed that the linearity assumptions were established to some extent. When using the results in this paper, it is important to confirm the dose–response relationship in preliminary experiments or in past experiments using analogous substances. It is necessary to examine, *ex post facto*, the linearity by displaying in figure or by linearity tests.

5.4. Group size

The group size must be an integer of at least 1. In addition, the total number of animals must be a comparatively small. Under these conditions, as in this paper, it is necessary to obtain the appropriate design using numerical calculations, separately considering a combination of possible group sizes. However, in order to generalize these results, it is useful to perform power calculations, taking the group size as a continuous value. When calculating the recommended ratio of the total number of animals in the single administration group to the group size of the simultaneous administration group, the results shown in Figure 5 are obtained.

When determining the appropriate group point of the simultaneous administration group, with the departure Δ from the additivity being constant, the highest power is obtained by setting the number of

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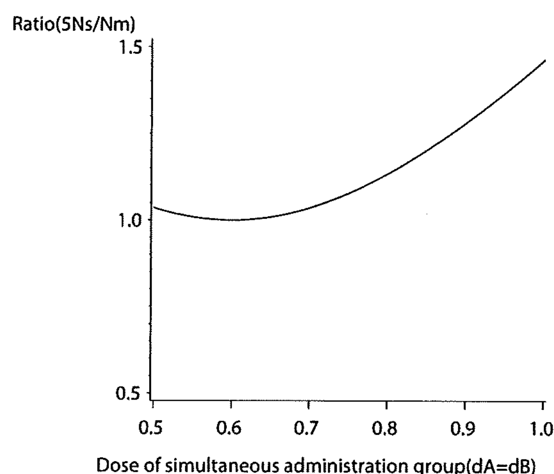

Figure 5. Recommended ratio of the group size between single administration group and simultaneous administration group

animals at a ratio of 1:1 between the single administration group and the simultaneous administration group.

When determining the simultaneous administration group on the boundary of the triangular region, a theoretical calculation shows that the allocation at a ratio of 1.464:1 is appropriate. In other words, it is best to set the total number of animals in the single administration group to be approximately 1.5 times the total number of animals in the simultaneous administration group. The reason is that because it is theoretically favorable for the accuracy of estimates based on additivity to be equal to that based on simultaneous administration.

5.5. Practical consideration on the recommended design

In the numerical examples of the previous section, we showed that the group point for the simultaneous administration on the boundary of the triangular region is not necessarily best and the recommended number of animals for the simultaneous administration group is considerably greater than those for single administration groups.

Although these results speciously imply that the design exemplified in the Subsection 4.1 should be replaced with the recommended design shown in this paper in future, we think it is not always true, because we have to take practical conditions into consideration, which were not incorporated in the assumptions to derive the recommended design.

One of them is the robustness or stability of the result of data analysis in such experiments. The situation is quite similar to that for the recommended design at linear regression analysis, for example, related to a single chemical experiment. Actually, if we can entirely assume the linearity of the dose–response relationship in regression analysis, the recommended design is to allocate a half of animals to the maximum dose and the remaining half to the minimum dose, while such design is really not adopted and animals are evenly allocated to uniformly distributed three or four doses probably to secure the robustness of the result of data analysis. Likewise, when the functional relationship of the Δ is not particularly clear, or when there is concern for the instability in squeezing the simultaneous administration group into one group in our experiments, the design with two or three simultaneous

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administration groups within the triangular region must be practical. Since the mathematical formulation in such condition could not be established up to present due to its difficult nature, we left it for future investigation.

5.6. *Other issues for future investigation*

When the number of test substances is 3 or more, there needs to be strict controls for the number of required animals. Therefore, a design must be determined by examining the design conditions in detail for each case. Under this condition, an investigation following the same approach as in this paper is necessary. This is another problem to be addressed in future research.

In this paper, we introduced a test statistic by using the unweighted least squares method. The weighted least squares method must be considered when the rules for the size of variance are understood from past research, such that variance linearly becomes larger along with the dose. The specific design in this instance is also to be investigated in the future.

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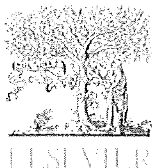

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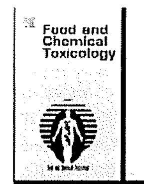

Carcinogenicity studies of 1,4-dioxane administered in drinking-water to rats and mice for 2 years

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ABSTRACT

The carcinogenicity of 1,4-dioxane was examined by giving groups of 50 F344/DuCrj rats and 50 Crj:BDF_1 mice of each sex 1,4-dioxane in the drinking-water for 2 years. The concentrations of 1,4-dioxane were 0 (control), 200, 1000 and 5000 ppm (wt./wt.) for rats and 0, 500, 2000 and 8000 ppm for mice. The highest dose levels did not exceed the maximum tolerated dose. In the rat, there was significant induction of nasal squamous cell carcinomas in females and hepatocellular adenomas and carcinomas in males and females, peritoneal mesotheliomas in males, and mammary gland adenomas in females. In the mouse, there was significant induction of hepatocellular tumors in males and females. Two nasal tumors occurring in the 8000 ppm-dosed groups were spontaneously rare and, thus, were attributed to 1,4-dioxane exposure. The present studies provided clear evidence of carcinogenicity in rats and mice. Lifetime cancer risk of humans exposed to 1,4-dioxane through drinking-water was quantitatively estimated with a non-threshold approach by application of a linearized multistage model to dose–carcinogenic response relationships, in addition to a threshold approach for estimation of the tolerable daily intake using no-observed- or lowest-observed-adverse-effect levels of the carcinogenic responses and uncertainty factors.

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1. Introduction

1,4-Dioxane is a commodity chemical used in large amounts by industry. Major uses of dioxane include stabilization of chlorinated solvents, solvent applications during the production of a wide variety of goods, and as a component of paints and other surface coatings (Lewis, 1993; O'Neil, 2006). The worldwide production volume of 1,4-dioxane was estimated to be 10,000 tons in 1995 (European Chemicals Bureau, 2002). Release of 1,4-dioxane into surface water from manufacturing and processing facilities in the USA was estimated to be 41 tons in 2004 (ATSDR, 2007). The annual production of 1,4-dioxane in Japan was reported to amount to 4500 tons in 2006 (Chemical Daily, 2008), and according to the Pollutant Release and Transfer Register Report from the Japan Ministry of the Environment (2008), 65 tons of 1,4-dioxane was released into public waters from the chemical industry in 2006. The Japan Ministry of the Environment (2002) reported that 1,4-dioxane was detected in numerous public waters of Japan in 2000, and at that time its concentrations ranged from 0.08 to 160 μg/l.

An epidemiological study showed no apparent excess of total cancer deaths in a small cohort of workers who were exposed to low levels of 1,4-dioxane in a manufacturing and processing facility (Buffler et al., 1978). However, several studies demonstrated that a long-term oral administration of 1,4-dioxane in the drinking-water induces hepatocellular and nasal tumors in rats and mice (Argus et al., 1965, 1973; Hoch-Ligeti et al., 1970; Kociba et al., 1974; NCI, 1978). Based on evidence in humans and experimental animals, the International Agency for Research on Cancer (IARC, 1999) has evaluated 1,4-dioxane as being a Group 2B carcinogen – it is demonstrably carcinogenic in animals, but there is not sufficient evidence to indicate that it is carcinogenic in humans – Group 2B substances are possible human carcinogens. Although almost all *in vitro* tests for genotoxic activity of 1,4-dioxane produced negative results (IARC, 1999), positive

Abbreviations: ATSDR, Agency for Toxic Substances and Disease Registry; GC, gas chromatography; GST-P, glutathione *S*-transferase placental form; H&E, hematoxylin and eosin; IARC, International Agency for Research on Cancer; JBRC, Japan Bioassay Research Center; LOAEL, lowest-observed-adverse-effect level; MOE, margin of exposure; MTD, maximum tolerated dose; NCI, National Cancer Institute; NOAEL, no-observed-adverse-effect level; NOS, not otherwise specified; NRC, National Research Council; OECD, Organization for Economic Co-operation and Development; TDI, tolerable daily intake; US EPA, United States Environmental Protection Agency; WHO, World Health Organization.

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genotoxicity of 1,4-dioxane has been reported with the *in vivo* mouse liver and bone marrow micronucleus assays (Mirkova, 1994; Morita and Hayashi, 1998; Roy et al., 2005). The United States Environmental Protection Agency (US EPA, 2008) estimated a lifetime cancer risk of 1 in 100,000 for people drinking-water contaminated with 30 μg/l 1,4-dioxane. The World Health Organization (WHO, 2008) has issued a guideline value for drinking-water quality of 50 μg/l 1,4-dioxane, based on both non-threshold and threshold approaches.

The present studies were intended to better characterize rodent carcinogenicity of 1,4-dioxane in the drinking-water for carcinogenic risk assessment of humans ingesting 1,4-dioxane-contaminated water, and to delineate dose–carcinogenic response relationships using one control and three dosed groups encompassing an appropriate range of dose levels that would allow to extrapolate to human exposure. The highest dose levels were determined not to exceed the maximum tolerated dose (MTD) according to the criteria set by IARC (Bannasch et al., 1986) and National Cancer Institute (NCI) (Sontag et al., 1976) for the 2-year bioassay study of rodent carcinogenicity. Some of the present results were preliminarily presented at the Second Asia–Pacific Symposium on Environmental and Occupational Health in 1993 (Yamazaki et al., 1994). In comparison with our preliminary report, however, we improved the diagnosis of pre- and neoplastic lesions in the liver according to the current diagnostic criteria (Mohr, 1997; Deschl et al., 2001). We estimated the lifetime cancer risk for 1,4-dioxane using a non-threshold approach of dose-carcinogenic response relationships based on positive *in vivo* genotoxic carcinogenicity, and compared those data with the safe drinking-water concentration derived from the tolerable daily intake (TDI) using no-observed-adverse-effect levels (NOAEL) or lowest-observed-adverse-effect level (LOAEL) of the carcinogenic responses and uncertainty factors based on a threshold approach for carcinogens that would interact indirectly with DNA.

2. Materials and methods

This study was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (OECD, 1981a) and the OECD Guideline for Testing of Chemicals 451 (Carcinogenicity Studies) (OECD, 1981b). The animals were cared for in accordance with guidelines for the care and use of laboratory animals (NRC, 1977). The present studies were approved by the ethics committee of the Japan Bioassay Research Center (JBRC).

2.1. Chemical

1,4-Dioxane (CAS Number 123-91-1, spectrometric grade, more than 99.0% pure, less than 0.3% water and less than 0.01% nonvolatile chemicals) was obtained in two different lots from Dojindo Laboratories (Kumamoto, Japan), and stored in a dark vessel at room temperature. Each lot of the test substance was analyzed for purity and stability by both infrared spectrometry (Hitachi 270-30, Hitachi Ltd., Ibaraki, Japan) and gas chromatography (GC) (GC-9A, Shimadzu Co., Kyoto, Japan) before and after its use. Neither decomposition products nor impurities were detected. No GC peak other than 1,4-dioxane was detected.

2.2. Preparation of 1,4-dioxane-formulated drinking-water and its stability

1,4-Dioxane was dissolved in deionized water at a target concentration of 0 (control), 200, 1000 or 5000 ppm (wt./wt.) for rats and 0, 500, 2000 or 8000 ppm for mice. The highest dose levels were chosen so as not to exceed the MTD, based on both growth rate and toxicity in our previous 13-week drinking-water studies (Kano et al., 2008). The 1,4-dioxane-formulated drinking-water was prepared twice a week and administered using a sipper bottle made of brown glass during a 3- or 4-day period. The volume of the sipper bottle was 200 ml for rats and 35 ml for mice. The concentrations of 1,4-dioxane in the drinking-water were determined at the time of preparation by GC, and found to be 90–114% of the target concentrations for rats and 94–111% for mice. The stability of the 1,4-dioxane in the drinking-water was examined 4 days after preparation of the 1,4-dioxane-formulated water and found to remain at 96–97% of the initial concentrations for rats and at 87–92% for mice.

2.3. Animals

Male and female F344/DuCrj rats and Crj:BDF_1 mice were purchased from Charles River Japan Inc. (Kanagawa, Japan) at 4 weeks of age. The animals were quarantined and acclimated for 2 weeks, and divided by stratified randomization into four body weight-matched groups, each consisting of 50 rats and 50 mice of each sex. An oral administration of 1,4-dioxane-formulated water to the animals was started at 6 weeks of age.

These animals were individually housed in stainless steel wire-mesh hanging cages (170W × 294D × 176H mm for rats and 112W × 212D × 120H mm for mice) under controlled environmental conditions (temperature of 24 ± 1 °C and a relative humidity of 50 ± 5%, with 15–17 room air changes/h). Fluorescent lighting was controlled automatically to provide a 12-h light/dark cycle. All animals were given basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and the assigned drinking-water *ad libitum*.

2.4. Experimental design and clinical observations

Groups of 50 rats and 50 mice of each sex were orally administered 1,4-dioxane in the drinking-water at the designated concentrations for 104 weeks (2 years). Groups of 50 rats and 50 mice of each sex serving as control received only deionized water for 2 years. The animals were observed daily for clinical signs and mortality. Body weight and water consumption were measured weekly for the first 14 weeks of the 2-year administration period and every 2 weeks thereafter. Food consumption was measured weekly for the first 14 weeks and every 4 weeks thereafter. Daily 1,4-dioxane intake was calculated as the concentration of 1,4-dioxane in drinking-water, multiplied by the volume of drinking-water consumed on a daily basis, and divided by the animal's body weight. A complete necropsy was performed on all animals, including those that were found dead or in a moribund state.

2.5. Macroscopic and histopathological examinations

Organs were examined macroscopically, and the tissues specified in the OECD test guideline (OECD, 1981b) and the entire respiratory tract including the nasal cavity were subjected to histopathologic examination. The organs and tissues were fixed in 10% neutral buffered formalin. The nasal cavity was decalcified in formic acid–formalin solution prior to trimming, and was transversely trimmed at three levels according to the procedure described in our previous paper (Nagano et al., 1997): at the level of the posterior edge of the upper incisor teeth (Level 1), at the incisive papilla (Level 2) and at the level of the anterior edge of the upper molar teeth (Level 3). The organs and tissues were embedded in paraffin and 5 μm-thick sections were prepared and stained with hematoxylin and eosin (H&E). The hepatic hyperplasia of rats and mice diagnosed in the previous report (Yamazaki et al., 1994) was re-examined histopathologically and changed to hepatocellular adenomas and altered hepatocellular foci including acidophilic, basophilic and clear cell foci in the present studies, according to the current diagnostic criteria of liver lesions in rats and mice (Mohr, 1997; Deschl et al., 2001).

2.6. Statistics and data analysis

Survival curves were plotted according to the method of Kaplan–Meier (Kaplan and Meier, 1958). The log-rank test (Peto et al., 1977) was used to test for a statistically significant difference in survival rate between any 1,4-dioxane-dosed group of either sex and the respective control group. Body and organ weights, and food and water consumption were analyzed by Dunnett's Test. Incidences of nonneoplastic lesions were analyzed by the chi-square test. Incidences of neoplastic lesions were analyzed for a dose–response relationship by Peto's test (Peto et al., 1980) and for a statistically significant difference from the concurrent control group by Fisher's exact test. A biologically meaningful increase in the incidence of rare tumors was evaluated by whether or not the observed incidence exceeded the maximum tumor incidence in the JBRC historical control data compiled from 2-year studies of rodent carcinogenicity conducted by the JBRC during a 21-year period from 1987 to 2007. Two-tailed testing was used for all statistical analyses except for Peto's test. In all cases, statistical analysis with p-values of 0.05 and 0.01 was performed and is indicated in the tables; a p-value of 0.05 was used for statistical significance.

NOAEL and LOAEL were determined according to the WHO definition (WHO, 1994).

3. Results

3.1. Rat study

3.1.1. Survival, food and water consumptions, chemical intake, and body and organ weights

Survival rates of the 5000 ppm-dosed males and females were significantly decreased (Fig. 1A and B), and the decreased survival rates were attributed to the increased number of deaths due to

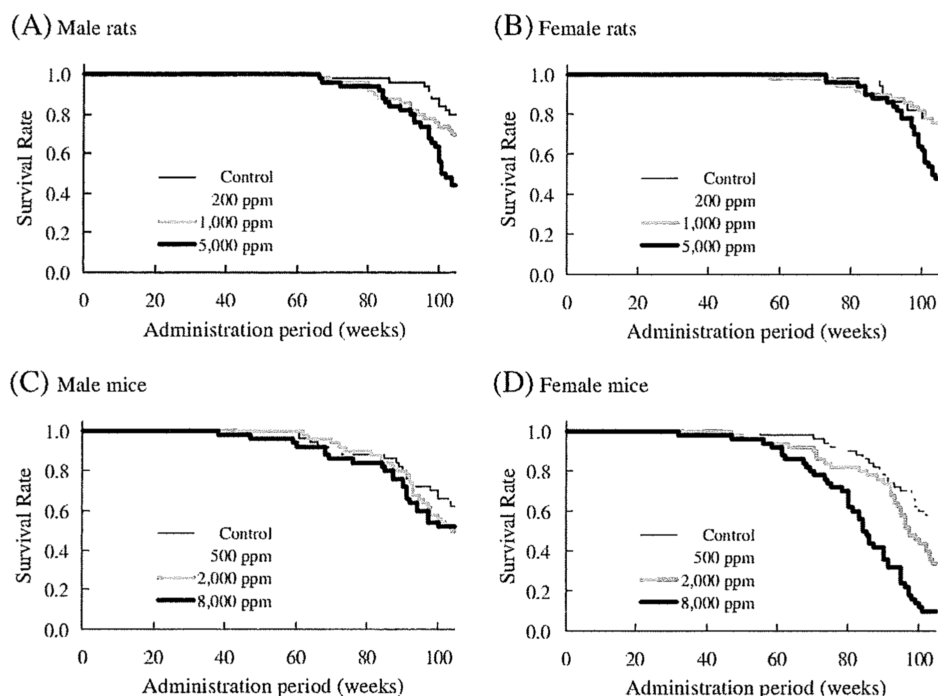

Fig. 1. Survival curves of rats (A and B) and mice (C and D) of each sex administered 1,4-dioxane in the drinking-water for 2 years.

nasal tumors and peritoneal mesotheliomas in the males and to nasal and hepatic tumors in the females.

Neither food nor water consumption was significantly decreased in any 1,4-dioxane-dosed group (Table 1). The estimated daily intake per body weight of 1,4-dioxane increased proportionally with dosage.

Growth rates of the males and females given 5000 ppm were significantly retarded (Fig. 2A and B), and the terminal body weights in these groups were significantly decreased (Table 2). The 20% decrease in the terminal body weight of the 5000 ppm-dosed females was attributed to the increased incidences of malignant tumors.

Table 1
Food consumption, water consumption and chemical intake of rats and mice orally administered 1,4-dioxane in the drinking-water for 2 years.

Group (ppm)	Control	200	1000	5000
(A) Rats				
Male				
Number of animals examined	50	50	50	50
Food consumption (g/day)	16.7 ± 0.7	16.6 ± 0.7	16.7 ± 0.6	16.4 ± 0.8
Water consumption (g/day)	20.6 ± 1.3	20.4 ± 1.2	20.8 ± 1.3	20.0 ± 1.2
1,4-Dioxane intake (mg/kg/day)	–	11 ± 1	55 ± 3	274 ± 18
Female				
Number of animals examined	50	50	50	50
Food consumption (g/day)	12.5 ± 0.6	12.7 ± 0.7	12.5 ± 0.5	12.5 ± 0.7
Water consumption (g/day)	19.1 ± 2.9	20.5 ± 3.2*	19.3 ± 2.8	19.0 ± 2.7
1,4-Dioxane intake (mg/kg/day)	–	18 ± 3	83 ± 14	429 ± 69
	Control	500	2000	8000
(B) Mice				
Male				
Number of animals examined	50	50	50	50
Food consumption (g/day)	4.4 ± 0.2	4.4 ± 0.2	4.4 ± 0.3	4.1 ± 0.2**
Water consumption (g/day)	4.2 ± 0.4	4.2 ± 0.3	4.1 ± 0.4	3.2 ± 0.3**
1,4-Dioxane intake (mg/kg/day)	–	49 ± 5	191 ± 21	677 ± 74
Female				
Number of animals examined	50	50	50	50
Food consumption (g/day)	4.1 ± 0.3	4.1 ± 0.3	4.2 ± 0.2	3.8 ± 0.2**
Water consumption (g/day)	4.3 ± 0.4	4.2 ± 0.5	4.3 ± 0.5	3.2 ± 0.2**
1,4-Dioxane intake (mg/kg/day)	–	66 ± 10	278 ± 40	964 ± 88

Values indicate mean ± SD.
* Significantly different at $p < 0.05$ by Dunnett's test.
** Significantly different at $p < 0.01$ by Dunnett's test.

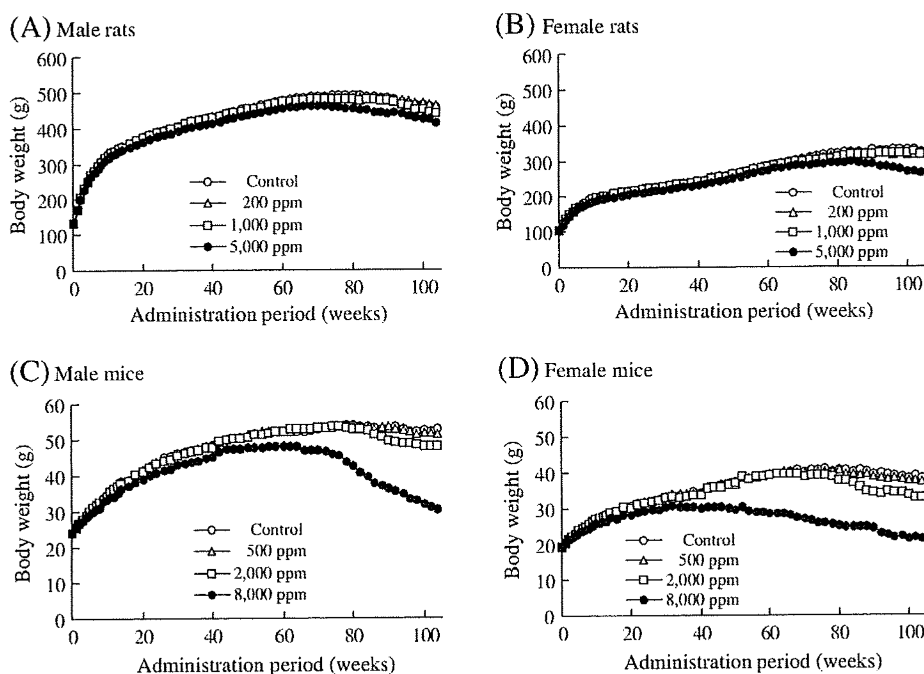

Fig. 2. Growth curves of rats (A and B) and mice (C and D) of each sex administered 1,4-dioxane in the drinking-water for 2 years.

Table 2
Terminal body weights and relative liver weights of rats and mice after oral administration of 1,4-dioxane in the drinking-water for 2 years.

Group (ppm)	Control	200	1000	5000
(A) Rats				
Male				
Number of animals examined[a]	40	45	35	22
Terminal body weight (g)	428 ± 36	433 ± 32	410 ± 53	391 ± 71**
Liver (%)	2.92 ± 0.31	3.03 ± 0.60	3.33 ± 0.52**	5.01 ± 1.10**
Female				
Number of animals examined[a]	38	37	38	24
Terminal body weight (g)	303 ± 41	301 ± 38	296 ± 29	242 ± 42**
Liver (%)	2.72 ± 0.69	2.63 ± 0.59	2.73 ± 0.41	7.35 ± 2.28**
	Control	500	2000	8000
(B) Mice				
Male				
Number of animals examined[a]	31	33	25	26
Terminal body weight (g)	48.7 ± 6.1	47.3 ± 6.8	44.1 ± 7.6*	27.0 ± 3.0**
Liver (%)	4.45 ± 2.65	4.93 ± 2.44	6.20 ± 4.33*	6.47 ± 2.57**
Female				
Number of animals examined[a]	29	29	17	5
Terminal body weight (g)	35.3 ± 5.1	33.8 ± 6.4	29.7 ± 4.7**	19.3 ± 2.8**
Liver (%)	4.48 ± 1.20	4.38 ± 1.38	5.11 ± 0.94	6.56 ± 1.96**

Values indicate mean ± SD.
[a] Number of animals examined are those which survived to the end of the 2-year administration period.
* Significantly different at $p < 0.05$ by Dunnett's test.
** Significantly different at $p < 0.01$ by Dunnett's test.

Relative liver weight was significantly increased in the 1000 and 5000 ppm-dosed males and in the 5000 ppm-dosed females (Table 2).

3.1.2. Histopathology

1,4-Dioxane-induced tumors occurred in the nasal cavity, liver, subcutis, mammary gland and peritoneum (Table 3). A dose-dependent increase in the incidences of nasal squamous cell carcinomas in males and females was also observed. The increase in nasal squamous cell carcinomas in the 5000 ppm-dosed females was statistically significant. Squamous cell carcinomas were morphologically well-differentiated and keratinized, and frequently exhibited invasive growth into the submucosal tissue and destruction of the nasal bone or septum. Nasal esthesioneuroepitheliomas, rhabdomyosarcomas and sarcomas NOS (not otherwise specified) were also observed. Since these nasal tumors have not been observed in the historical control data of the JBRC (no cases in 2149 male rats in 43 carcinogenicity studies or in 1997 female rats in 40 carcinogenicity studies), they were judged to be compound-related. The squamous cell

Table 3
The number of rats bearing selected lesions in the nasal cavity, liver, subcutis, mammary gland and peritoneum after oral administration of 1,4-dioxane in the drinking-water for 2 years.

	Male					Female				
Group (ppm)	Control	200	1000	5000	Peto test	Control	200	1000	5000	Peto test
Number of animals examined	50	50	50	50		50	50	50	50	
Nasal cavity										
Squamous cell carcinoma	0	0	0	3	††	0	0	0	$7^{\#\#}$	††
Esthesioneuroepithelioma	0	0	0	1		0	0	0	1	
Rhabdomyosarcoma	0	0	0	1		0	0	0	0	
Sarcoma NOS	0	0	0	2		0	0	0	0	
Respiratory epithelium										
Nuclear enlargement	0	0	0	26^{**}		0	0	0	13^{**}	
Squamous cell metaplasia	0	0	0	31^{**}		0	0	0	35^{**}	
Squamous cell hyperplasia	0	0	0	2		0	0	0	5	
Olfactory epithelium										
Nuclear enlargement	0	0	5	38^{**}		0	0	28^{**}	39^{**}	
Liver										
Hepatocellular adenoma	3	4	7	$32^{\#\#}$	††	3	1	6	$48^{\#\#}$	††
Hepatocellular carcinoma	0	0	0	$14^{\#\#}$	††	0	0	0	$10^{\#\#}$	††
Hepatocellular adenoma or carcinoma	3	4	7	$39^{\#\#}$	††	3	1	6	$48^{\#\#}$	††
Altered hepatocellular foci										
Acidophilic cell foci	12	8	7	5		1	1	1	1	
Basophilic cell foci	7	11	8	16^{*}		23	27	31	8^{**}	
Clear cell foci	3	3	9	8		1	1	5	4	
Mixed cell foci	2	8	14^{**}	13^{**}		1	1	3	11^{*}	
Subcutis										
Fibroma	5	3	5	12	††	0	2	1	0	
Mammary gland										
Adenoma	0	1	2	2		6	7	10	$16^{\#}$	††
Fibroadenoma	1	1	0	4	††	3	2	1	3	
Adenoma or Fibroadenoma	1	2	2	6	††	8	8	11	$18^{\#}$	††
Peritoneum										
Mesothelioma	2	2	5	$28^{\#\#}$	††	1	0	0	0	

Note: Lesions in animals which became moribund or died before the end of the 2-year administration period were also scored.
\# Significantly different from control at $p < 0.05$ by Fisher's exact test.
\#\# Significantly different from control at $p < 0.01$ by Fisher's exact test.
* Significantly different at $p < 0.05$ by chi-square test.
** Significantly different at $p < 0.01$ by chi-square test.
†† Significantly different at $p < 0.01$ by Peto's test.

carcinomas arose from the dorsal wall and septum at Level 1 through 3 (Table 4). The other types of nasal tumors were located in the dorsal wall, septum and turbinate at Levels 2 and 3.

Proliferative and preneoplastic lesions (squamous cell hyperplasias and squamous cell metaplasias) in the nasal cavity were observed in the 5000 ppm-dosed groups (Table 3). Squamous cell hyperplasia was characterized by a focal increase in the number of the epithelial cell layers in the squamous epithelium to five or more layers together with keratinization. Squamous cell metaplasia was characterized by replacement of respiratory epithelium with squamous epithelium with or without keratinization. Some of the squamous cell metaplasia was accompanied by atypia that was characterized by irregular size and shape of the cells and irregular arrangement of the cell layer, and were regarded as potentially preneoplastic lesions (Brown, 1990; Monticello et al., 1990). Furthermore, the incidence of nuclear enlargement was significantly

Table 4
The location of nasal tumors in rats and mice after oral administration of 1,4-dioxane in the drinking-water for 2 years.

	Male			Female			
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3	Other
Rats							
Squamous cell carcinoma	2		1	1	4		2[a]
Esthesioneuroepithelioma		1				1	
Rhabdomyosarcoma			1				
Sarcoma NOS		2					
Mice							
Adenocarcinoma						1	
Esthesioneuroepithelioma			1				

Note: Lesions in animals which became moribund or died before the end of the 2-year administration period were also scored.
Level 1: Level at the posterior edge of the upper incisor teeth.
Level 2: Level at the incisive papilla.
Level 3: Level at the anterior edge of the upper molar teeth. The values indicate numbers of animals bearing a nasal tumor which originated from Level 1, 2 or 3.
[a] One of the two rats had a relatively large tumor occupying the area at Levels 1 and 2, and another had a large tumor infiltrating over the entire region at Levels 1, 2 and 3.

increased in both respiratory and olfactory epithelia of the 5000 ppm-dosed males and females and in the olfactory epithelium of the 1000 ppm-dosed females.

Hepatocellular adenomas and carcinomas occurred dose-dependently, and statistically significant increases occurred in the 5000 ppm-dosed males and females. Since the incidences of hepatocellular adenomas in the 1000 ppm-dosed males and females (14% and 12%, respectively) exceeded the maximum incidences of the JBRC historical control data for hepatocellular adenomas (41 cases, 1.9%, in 2149 male rats with a maximum incidence of 8%, and 22 cases, 1.1%, in 1977 female rats with a maximum incidence of 6.1%), the hepatocellular adenomas occurring in the 1000 ppm-dosed males and females were judged to be compound-related. A significant increase in the incidence of altered hepatocellular foci was observed in the 1000 and 5000 ppm-dosed males and in the 5000 ppm-dosed females.

Subcutaneous fibromas occurred dose-dependently in the males, although the tumor incidences were not statistically significant. In the mammary gland, adenomas and fibroadenomas occurred dose-dependently in the females and males, respectively, and the incidence of adenomas was significantly increased in the 5000 ppm-dosed females. Peritoneal mesotheliomas occurred dose-dependently in the males, and a statistically significant increase occurred in the 5000 ppm-dosed group.

3.2. Mouse study

3.2.1. Survival, food and water consumptions, chemical intake, and body and organ weights

There was no difference in survival rate between any 1,4-dioxane-dosed male group and the male control (Fig. 1C). The female groups given 2000 and 8000 ppm, on the other hand, did show significantly decreased survival rates (Fig. 1D). The decreased survival rates of these two groups were attributed to the increased number of deaths due to hepatic tumors.

Both food and water consumption were significantly decreased in the 8000 ppm-dosed males and females (Table 1). The estimated daily intake per body weight of 1,4-dioxane in the males and females given 8000 ppm did not increase proportionally as compared with that of the males and females given 500 or 2000 ppm 1,4-dioxane. This decreased 1,4-dioxane intake was attributed to the decreased water consumption in the 8000 ppm-dosed males and females.

Growth rates of the males and females given 2000 and 8000 ppm were significantly retarded (Fig. 2C and D), and the terminal body weights in these groups were significantly decreased (Table 2). The decreased body weights were attributed to the increased incidences of malignant liver tumors.

Relative liver weight was significantly increased in the 8000 ppm-dosed group of both sexes and in the 2000 ppm-dosed males (Table 2).

3.2.2. Histopathology

1,4-Dioxane-induced tumors occurred in the nasal cavity and liver (Table 5). One case each of a nasal esthesioneuroepithelioma and a nasal adenocarcinoma occurred in an 8000 ppm-dosed male and female mouse, respectively. Since these nasal tumors have not been observed in the historical control data of the JBRC (no case of esthesioneuroepithelioma in 1846 male mice and no case of adenocarcinoma in 1847 female mice in 37 carcinogenicity studies), they were judged to be compound-related. These nasal tumors were located in the ethmoturbinate at Level 3 (Table 4). The incidence of nuclear enlargement in the nasal cavity was significantly increased in both respiratory and olfactory epithelia of the 8000 ppm-dosed males and females, and in the olfactory epithelium of the 2000 ppm-dosed males and females.

Hepatocellular carcinomas occurred dose-dependently, and their incidences were significantly increased in the 8000 ppm-dosed males and in all 1,4-dioxane-dosed female groups (Table 5). A significant induction of hepatocellular adenomas occurred in the 2000 ppm-dosed males and in the 500 ppm and 2000 ppm-dosed females.

4. Discussion

In this study with rats, a 2-year oral administration of 1,4-dioxane in the drinking-water was found to induce squamous cell carcinomas in the nasal cavity of females, hepatocellular adenomas and carcinomas of both males and females, mesotheliomas in the peritoneum of males and adenomas in the mammary gland of females. The incidences of these tumors were dose-dependent and statistically significant. Dose-dependent increases in the incidences of squamous cell carcinomas in the nasal cavity, fibromas in the subcutis and fibroadenomas in the mammary gland was also noted in male rats, although the increases in these tumor incidences were not statistically significant. The spontaneously rare nasal tumors, esthesioneuroepithelioma, rhabdomyosarcoma and sarcoma NOS, that occurred in rats given the highest dose of 1,4-dioxane were judged to be compound-related, since these nasal tumors have not been observed in the historical control data of the JBRC.

In mice, 1,4-dioxane was found to induce hepatocellular adenomas and carcinomas in both males and females. The incidences of these liver tumors were dose-dependent and statistically significant. Spontaneously rare nasal tumors, esthesioneuroepithelioma and adenocarcinoma, were also observed in animals given the highest dose of 1,4-dioxane and were judged to be compound-related, since these nasal tumors have not been observed in the historical control data of the JBRC.

The carcinogenic responses of rats and mice found in these studies are consistent with the reported findings that a long-term oral administration of 1,4-dioxane in the drinking-water induced nasal squamous cell carcinoma and hepatocellular carcinoma in male and female rats (Argus et al., 1965, 1973; Hoch-Ligeti et al., 1970; Kociba et al., 1974; NCI, 1978). Rare nasal tumors were also observed in male and female rats and mice (adenocarcinomas in male and female rats and mice and rhabdomyomas in male rats) in the NCI study (1978), although the incidences of the rare nasal tumors were not compared with those from the NCI's historical control data.

Squamous cell hyperplasias and squamous cell metaplasias with atypia, both of which were observed in the 1,4-dioxane-dosed rats of both sexes, can be regarded as preneoplastic and potentially preneoplastic lesions (Brown, 1990; Monticello et al., 1990), respectively. The significantly increased incidence of nuclear enlargement of epithelial cells in the nasal cavity was noted in the rats and mice of both sexes given the high and middle dose levels except in the 1000 ppm-dosed male rats, and the enlarged nuclei were reported to be tumor-related (Grant and Grasso, 1978; Fowlie et al., 1990). The nuclear enlargement of nasal epithelial cells was observed in the rats and mice given 1,4-dioxane for 13 weeks (Kano et al., 2008). As a preneoplastic lesion of hepatic tumors, altered hepatocellular foci were increased in the male and female rats given 1,4-dioxane for 2 years. We previously confirmed that glutathione *S*-transferase placental form (GST-P) positive, hepatocellular foci occurred in the rats given 1,4-dioxane-formulated drinking-water for 13 weeks (Kano et al., 2008). The GST-P positive, hepatocellular foci are thought to allowed to predict rat hepatocarcinogenicity with high probability (Ito et al., 2000).

Table 5
The number of mice bearing selected lesions in the nasal cavity and liver after oral administration of 1,4-dioxane in the drinking-water for 2 years.

	Male					Female				
Group (ppm)	Control	500	2000	8000	Peto test	Control	500	2000	8000	Peto test
Number of animals examined	50	50	50	50		50	50	50	50	
Nasal cavity										
Adenocarcinoma	0	0	0	0		0	0	0	1	
Esthesioneuroepithelioma	0	0	0	1		0	0	0	0	
Respiratory epithelium										
Nuclear enlargement	0	0	0	31**		0	0	0	41**	
Olfactory epithelium										
Nuclear enlargement	0	0	9**	49**		0	0	41**	33**	
Liver										
Hepatocellular adenoma	9	17	23##	11		5	31##	20##	3	
Hepatocellular carcinoma	15	20	23	36##	††	0	6#	30##	45##	††
Hepatocellular adenoma or carcinoma	23	31	37#	40##	††	5	35##	41##	46##	††

Note: Lesions in animals which became moribund or died before the end of the 2-year administration period were also scored.
Significantly different from control at $p < 0.05$ by Fisher's exact test.
Significantly different from control at $p < 0.01$ by Fisher's exact test.
** Significantly different at $p < 0.01$ by chi-square test.
†† Significantly different at $p < 0.01$ by Peto's test.

Some differences in the carcinogenicity of 1,4-dioxane were found between the present studies and the earlier studies mentioned above. First, in these studies, additional types of tumors are reported: in males subcutaneous fibromas, mammary gland fibroadenomas and peritoneal mesotheliomas were observed, and in females mammary gland adenomas were observed. These tumors were induced dose-dependently, and increases in the incidence of mesotheliomas in males and mammary adenomas in females were statistically significant. Second, in the present studies, we used a lower dose range of 1,4-dioxane, predicting that the MTD level existed below 10,000 ppm (1.0%), and found significant induction of hepatocellular tumors at lower dose levels. On the other hand, the NCI study (1978) showed induction of hepatocellular tumors in the 5000 and 10,000 ppm-dosed female rats and mice of both sexes. Thus, the observed dose–carcinogenic response relationships found in the present studies allow extrapolation to relevantly lower levels of environmental 1,4-dioxane.

Rat nasal tumors induced by the administration of 1,4-dioxane in the drinking-water have been questioned for relevance to humans. Reitz et al. (1990), Goldsworthy et al. (1991) and Sweeney et al. (2008) suggest that the carcinogenic effect observed in rats is due to direct contact of anterior nasal tissue with 1,4-dioxane-containing water and is not relevant to human exposure, since human nasal tissue does not come in direct contact with the drinking-water during normal drinking activity. The suggested irrelevance of 1,4-dioxane-formulated water-induced rat nasal tumors to human exposure is favored by Torkelson et al.'s study (1974) that nasal tumors were not observed in rats exposed to 1,4-dioxane vapor at 111 ppm for 2 years. However, Kasai et al. (2009) demonstrated that inhalation exposure of male F344 rats to 1,4-dioxane vapor at 1250 ppm for 2 years induces squamous cell carcinoma, squamous cell hyperplasia, squamous cell metaplasia and nuclear enlargement in the nasal cavity. In the present studies, we found a wide distribution of nasal tumors over the entire region of the nasal cavity of rats (Table 4), and this is likely to result from exposure of the nasal tissue, after gastrointestinal absorption of orally administered 1,4-dioxane, to circulatory and exhalatory 1,4-dioxane or possibly to its reactive metabolites. It is notable that P450-2E1 isozymes which are involved in the metabolism of 1,4-dioxane are known to be enriched in the nasal mucosa, including the respiratory and olfactory epithelia (Bond, 1993; Nannelli et al., 2005), allowing exposure of nasal tissue at Levels 2 and 3 to reactive metabolites of 1,4-dioxane to occur. Although we can not rule out the possibility of repeated splashing of 1,4-dioxane-formulated drinking-water into the nose, it might not be rational to eliminate 1,4-dioxane-induced nasal tumors of rats as being irrelevant to human exposure. Indeed, squamous cell carcinoma in the nasal cavity was reported to occur in workers exposed, possibly by inhalation, to occupational agents in a nickel refinery (Torjussen et al., 1979) and in the furniture, boot and shoe industries (Acheson et al., 1968; Acheson, 1976).

The genotoxicity of 1,4-dioxane is an important determinant for carcinogen risk assessment. 1,4-Dioxane is negative in various *in vitro* assays such as a bacterial reverse mutation assay and cultured mammalian cell assays (IARC, 1999; Morita and Hayashi, 1998), but is positive in *in vivo* mouse liver and bone marrow micronucleus assays. Mirkova (1994) reported the induction of micronuclei by 1,4-dioxane with the bone marrow erythrocytes of mice. Morita and Hayashi (1998) demonstrated with a liver micronucleus assay that a single administration of 1,4-dioxane by oral gavage increases the frequency of micronucleated hepatocytes in the partially hepatectomized mice. Furthermore, Roy et al. (2005) reported a positive result on both liver and bone marrow of young CD-1 mice using CREST staining or pancentromeric FISH, showing that 80–90% of the micronuclei of both liver and bone marrow originated from chromosome breakage. Therefore, an *in vivo* genotoxic mode of action was suggested to operate in the 1,4-dioxane-induced carcinogenesis. However, this *in vivo* mode of action is considered not to rule out a possibility that the carcinogenesis is causally related to indirect interaction of 1,4-dioxane with DNA *in vivo* such as aneuploidy and alterations of mitosis and cell cycle resulting in genomic instability, rather than to the direct reactivity with DNA such as DNA adduct and breakage (US EPA, 2005).

The present dose–carcinogenic responses are characterized by use of two rodent species of both sexes, an appropriate range of three dose levels from 200 to 5000 ppm for rats and from 500 to 8000 ppm for mice, which would allow to extrapolate to environmentally relevant levels of 1,4-dioxane because the highest dose levels were below the MTD level. Diagnostic improvement from the hepatic hyperplasia in our preliminary report (Yamazaki et al., 1994) to the altered hepatocellular foci and hepatocellular adenomas in the present studies according to the current criteria (Mohr, 1997; Deschl et al., 2001) was found to increase the incidences of hepatocellular adenomas in the 1,4-dioxane-dosed groups, resulting in the definite dose–hepatocarcinogenic response

relationships as compared with those in our preliminary report. On the other hand, higher dose levels spanning from 5000 to 18,000 ppm 1,4-dioxane have been used in the water-drinking carcinogenicity studies using rats (Argus et al., 1965, 1973; Hoch-Ligeti et al., 1970) and using both rats and mice (NCI, 1978), whereas a wide range of dose levels of 100, 1000 and 10,000 ppm was given only the rat in Kociba et al.'s study (1974). Therefore, quantitative carcinogenic risk assessment of humans ingesting 1,4-dioxane-contaminated drinking-water can be estimated as a genotoxic carcinogen by application of these dose–carcinogenic responses of rats and mice to a non-threshold approach using the US EPA software program of a linearized multistage model (US EPA, 2001). In the present studies, only the nasal squamous cell carcinomas in female rats and the hepatocellular tumors in male rats and female mice were used to quantitatively estimate human cancer risk, since these nasal and hepatic tumors are of established relevancy to the human cancer risk as compared with the other types of 1,4-dioxane-induced tumors. It has been recognized that the peritoneal mesothelioma is a commonly observed, spontaneous neoplasm of male F344 rats arising from the tunica vaginalis (Hall, 1990), and that the subcutis fibroma and mammary gland adenoma and fibroadenoma are benign, commonly observed and spontaneous neoplasms of F344 rats (Boorman et al., 1990; Elwell et al., 1990). The estimated daily intake of 1,4-dioxane per body weight (mg/kg) was used as a dosage parameter without further correction to the body surface area, instead of the observed drinking-water concentration of 1,4-dioxane. The calculated cancer slope factors resulted in 4.7×10^{-4} $(mg/kg/day)^{-1}$ for nasal squamous cell carcinomas in female rats, 5.5×10^{-3} $(mg/kg/day)^{-1}$ for hepatocellular tumors in male rats and 5.1×10^{-3} $(mg/kg/day)^{-1}$ for hepatocellular tumors in female mice. Assuming that a 70-kg human consumes 2 l of water per day, the drinking-water concentrations at an upper-bound excess lifetime cancer risk of 1 in 100,000 corresponding to these cancer slope factors are calculated as follows: 745 μg/l using nasal squamous cell carcinomas data from female rats; 64 μg/l using from hepatocellular tumors data from male rats; and 68 μg/l from hepatocellular tumors data from female mice.

In this context, using a threshold approach that indirect interaction of 1,4-dioxane with DNA is involved in the carcinogenesis, we also derived the TDIs of 1,4-dioxane from the NOAEL or LOAEL for the carcinogenic endpoint and the total uncertainty factor. In the present studies, a NOAEL was 83 mg/kg/day for nasal squamous cell carcinomas in female rats and 11 mg/kg/day for hepatocellular tumors in male rats, while a LOAEL was 66 mg/kg/day for hepatocellular tumors in female mice. Using 1000 for the total uncertainty factor of non-genotoxic carcinogenicity for NOAEL and 10,000 for LOAEL including an additional uncertainty factor of 10 for LOAEL to NOAEL extrapolation (WHO, 1990), the TDIs resulted in 83, 11 and 6.6 μg/kg/day for the nasal squamous cell carcinoma in female rats, hepatocellular tumors in male rats and hepatocellular tumors in female mice, respectively. Assuming a fraction of the TDI allocated to the drinking-water to be 10% (WHO, 2008) and a water consumption rate of 2 l/day in a 70-kg human, the safe drinking-water concentrations derived from the TDIs were estimated to be 291, 39 and 23 μg/l, indicating that these concentrations derived from the TDIs are twofold lower than those from the multistage model. US EPA (2008) derived the value of 30 μg/l for estimated lifetime cancer risk of 1 in 100,000 by application of a linearized multistage model to the NCI data (NCI, 1978) of rat nasal tumors. WHO (2008) derived the guideline value of 50 μg/l from both the linearized multistage model for cancer risk using nasal and hepatic tumors of rats and the threshold approach of TDI using a NOAEL for an endpoint of non-genotoxic carcinogenicity and a total of uncertainty factors of 1000 (WHO, 1994). The safe drinking-water concentrations of 1,4-dioxane derived from both the non-threshold and threshold approaches in the present studies were found to be approximately equal to the US EPA's value (US EPA, 2008) and WHO's guideline value (WHO, 2008), except for our value from the nasal tumor data. Therefore, the present results would provide additional evidence for the safe drinking-water concentration of 1,4-dioxane on the basis of both non-threshold and threshold approaches using the dose-hepatocarcinogenic responses in rats and mice, and validate the guideline values proposed for human cancer risk by the US EPA (2008) and WHO (2008). Furthermore, a margin of exposure (MOE) (US EPA, 1996), which is a ratio between the NOAEL (11 and 83 mg/kg/day) for the carcinogenic endpoint and the estimated intake (4.6 μg/kg/day) resulting from exposure of a 70-kg human consuming water of 2 l/day to 1,4-dioxane at a reported concentration of 160 μg/l (Japan Ministry of the Environment, 2002), was found to range from 2400 to 18,000. As described by Gold et al. (2003), comparison of the MOE values with the total uncertainty factors suggests that the safe drinking-water concentrations derived from the threshold approach of TDI are also adequately protective of public health for cancer effects.

In conclusion, clear evidence of carcinogenicity was provided by a 2-year oral administration of 1,4-dioxane-formulated drinking-water to rats and mice. Dose-dependent increases of malignant nasal and peritoneal tumors and benign mammary gland and subcutaneous tumors in rats, as well as malignant and benign hepatocellular tumors in both rats and mice were observed. Dose–carcinogenic response relationships for nasal and hepatocellular tumors were used as data of relevancy to human risk to quantitatively estimate lifetime cancer risk of humans exposed to 1,4-dioxane through water-drinking using a non-threshold approach based on the *in vivo* genotoxic mode of action and the linearized multistage model. The drinking-water concentrations at an upper-bound excess lifetime cancer risk of 1 in 100,000 were found to be 745, 64 and 68 μg/l, derived from the nasal squamous cell carcinoma in female rats, the hepatocellular tumors in male rats and the hepatocellular tumors in female mice, respectively. Also, a threshold approach of the TDIs using NOAEL and LOAEL for the carcinogenic endpoint having indirect interaction with DNA and the total uncertainty factors allowed to estimate the safe drinking-water concentrations of 1,4-dioxane, which were twofold lower than those with the non-threshold approach.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Letter

Design and performance of a system for blood collection of rats under whole-body inhalation exposure

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ABSTRACT — In order to obtain basic risk assessment data on human health exposure to volatile organic compound (VOC) vapor by inhalation, a whole-body inhalation exposure system which allows blood collection during the exposure period was designed. The system was tested using chloroform as a model VOC. Chloroform vapor, sampled from the supply-header, animal-chambers and exhaust-header, remained constant in this system with variations in its concentration being less than 2%; flow rate of the vapor through the system was also constant. Rats were exposed to chloroform vapor and blood collected from the tail during exposure to the chloroform vapor. The chloroform concentration in the blood increased during the initial 60 min of exposure, and afterwards its concentration remained at about 2 µg/ml from 60 to 360 min. In conclusion, our design allows blood to be collected from individual rats during exposure by inhalation to test VOCs and changes in the blood concentration of the VOC during exposure to be assessed.

Key words: Whole-body inhalation exposure system, Blood collection, Rats, Chloroform

INTRODUCTION

Inhalation exposure studies on animals using volatile organic compounds (VOCs) have delineated dose-response relationships to determine effective endpoints for the risk assessment of people exposed to VOC vapor by inhalation. The vapor concentrations in most of these studies is used as the endpoint dose when determining the dose-response effects. However, it is the internal dose, i.e., the concentration of the VOC in the blood, and factors such as time-course changes in the blood concentration of the VOC which is usually more directly related to many of the adverse health effects of a particular VOC.

Blood collection from animals during the inhalation exposure period has been reported, but only when using a nose-only inhalation exposure system (CERI News, 2005). Blood concentrations of inhaled chemicals using whole-body inhalation systems have been described in some reports (Kaneko *et al.*, 1995; Wang *et al.*, 1995; Hori *et al.*, 1999), but the blood concentration data was obtained only after termination of inhalation exposure period. For whole-body inhalation exposure studies, the animals are individually housed in an inhalation exposure chamber and are exposed to the chemical substance; under these whole-body exposure conditions, it is impossible to collect the blood from the animal during the inhalation exposure period. Therefore, it is difficult to obtain blood concentration data that accurately corresponds to the exposure concentration.

We have designed a whole-body inhalation exposure system that allows blood to be collected from the tails of exposed rats during the inhalation exposure period. The system was verified using chloroform as a model VOC: chloroform is found in both outdoor and indoor air (International Programme on Chemical Safety, 1994; International Agency for Research on Cancer, 1999), and is a carcinogen (Yamamoto *et al.*, 2002; Nagano *et al.*, 2006).

MATERIALS AND METHODS

Whole-body inhalation exposure system

The system is composed of two parts: a whole-body inhalation apparatus in which the rats are exposed to chemical vapors and a blood-collection module in which the rats are housed during exposure and allows blood to be collected from the tail.

The whole-body inhalation apparatus is shown in

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Fig. 1. A chemical vapor-air mixture is generated by bubbling clean air through the test VOC. The vapor is supplied to the animal chambers via the supply-header and vented via the exhaust-header. A flow-meter and flow-control-valve are attached to each animal-chamber to control the flow rate of the chemical vapor-air mixture through the animal-chamber. The blood-collection module is inserted into the animal-chamber and is held in place by a holder-nut. The inhalation apparatus has 9 vapor sampling ports: one at each end of the supply-header (S-1 and S-2), one in each animal-chamber (A-1-6), and one in the exhaust-header (E-1). The supply-header and animal-chamber are made of acrylic acid resin, and the holder-nut and exhaust-header are made of poly vinyl chloride resin.

The blood-collection module is shown in Fig. 2. This module is composed of an animal-housing assembly and a tail-holder assembly. The animal-housing assembly consists of an animal-holder made of acrylic acid resin equipped with a dome type head-cover. The animal-holder has protruding circular fins to keep it from moving after it is placed within the whole-body inhalation apparatus. The tail-holder assembly is composed of a silicon-septum made of silicon rubber and a backup-plate made of stainless steel. The silicon-septum forms an airtight gasket between the backup-plate and the animal-holder to prevent chemical vapor leakage. A hole in the silicon-septum (position X in Figs. 2 and 3) allows for protrusion of the tail for blood collection and is smaller than the tail root of the rat to prevent vapor leakage. The animal-housing and tail-holder assembly are held firmly together by the holder-nut which also holds the blood-collection module in place in the whole-body inhalation apparatus.

The test animal is housed and blood collected as follows. The rat is placed in the animal-holder and the head-cover moved down the slide line to fit its body size. The head-cover is fixed in place with the head-cover screw and the animal-housing assembly is placed into the whole-body inhalation apparatus. Finally, the rat's tail is passed through the hole of the tail-holder assembly, and the tail-holder, the animal-housing assembly and the whole-body inhalation apparatus are clamped firmly together with the holder-nut. The rat is exposed to vapor and blood is collected from the tail vein as shown in Fig. 3.

Temperature relative humidity of the system is measured by thermo-hygro meter at 6 min intervals, and maintained in a range of 22 ± 3°C and 51 ± 8%, respectively. The flow rate of air through the animal-chamber is monitored by flow-meter during the inhalation exposure time, and controlled by a flow-control-valve within a range of 250 to 260 ml/min.

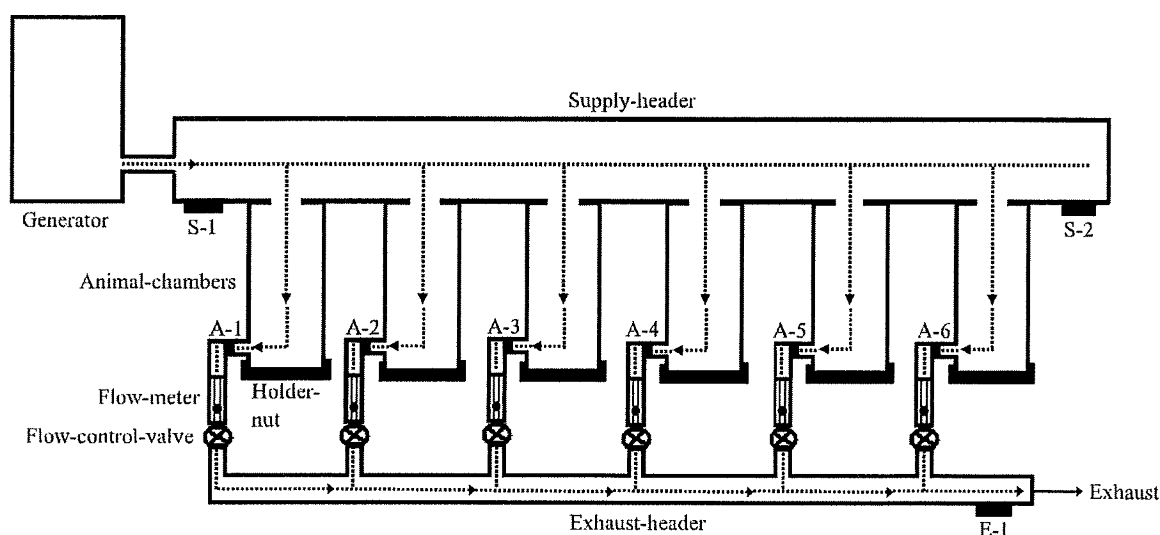

Fig. 1. Schematic diagram of whole-body inhalation apparatus.
S-1 and 2: sampling ports of supply-header, A-1-6: sampling ports of animal-chambers,
E-1: sampling port of exhaust-header.

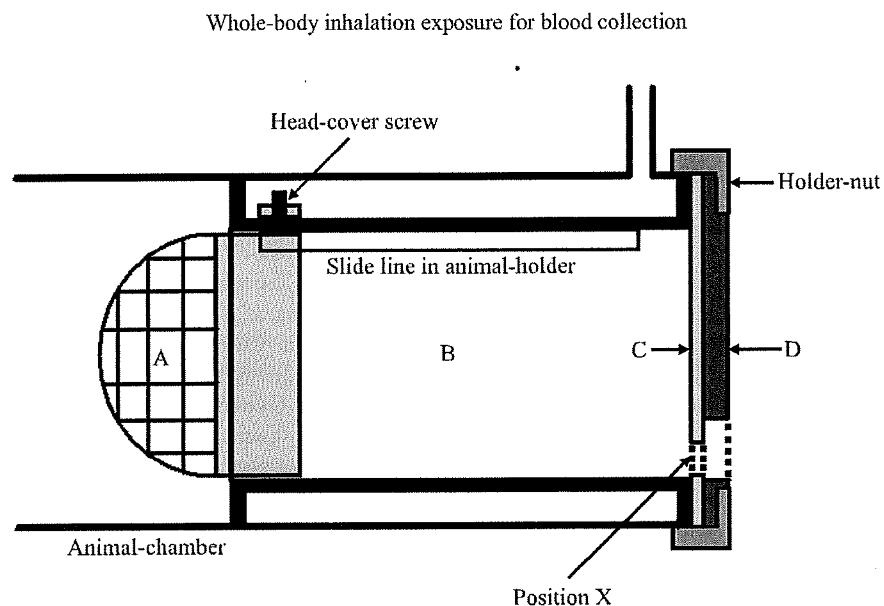

Fig. 2. Blood-collection module of whole-body inhalation exposure system.
Animal-housing assembly (A: Head-cover, B: Animal-holder) and tail-holder assembly (C: Silicon-septum, D: Backup-plate).

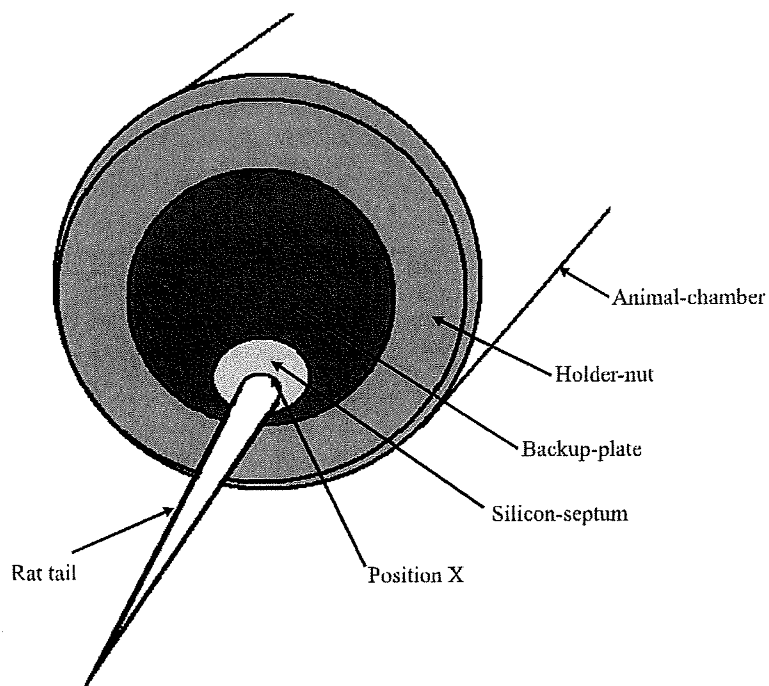

Fig. 3. Rat tail protruding through tail-holder assembly when blood-collection module is in place in whole-body inhalation exposure system.

Chemicals

Chloroform (purity greater than 99.0%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For the present study, chloroform vapor at a concentration of 100 ppm (v/v) was generated by bubbling clean air through liquid chloroform.

Animals

Male Crl:CD (SD) rats (SPF) 9 weeks of age were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Their body weights ranged from 341 to 368 g. Experiments were initiated after a one-week acclimation period. The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and the present study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC).

Study design

The performance of the whole-body inhalation exposure system was analyzed by the following 3 studies.

1. A performance study of the system without rats (study 1). The performance of the system without rats was analyzed by measuring chloroform vapor concentration and flow rate over the course of a single exposure day.
2. Confirmation of the performance of the system with rats (study 2). System performance with rats was analyzed by measuring chloroform vapor concentration and flow rate in the whole-body inhalation apparatus housing 6 rats over the course of a single exposure day. In addition, leakage of chloroform vapor from the tail-holder assembly of each animal-chamber was examined.
3. Use the system to analyze the concentration chloroform in the blood of test animals during the exposure to chloroform vapor (study 3). Six rats was were exposed to chloroform vapor (100 ppm) for 6 hr, and blood collected at 1, 30, 60, 120, 180, 240, 300 and 360 min from the start of exposure.

Analysis of chloroform vapor concentration in supply-header, animal-chamber and exhaust-header

Air in the supply-header, animal-chambers and exhaust-header was sampled using a RT731 (GL Sciences, Tokyo, Japan) every 15 min. Air at the rat tail outlet was sampled by a gas-tight syringe every 15 min. Chloroform vapor concentration was assessed by gas chromatography (GC) using Agilent Technologies 6890 (Agilent Technologies, Santa Clara, CA, USA) gas chromatography system (column, J&W DB-1 0.53 mmϕ × 5 m; oven temperature, 40°C; injection and detection temperature, 200°C; detector, FID; carrier gas, helium at 7.5 ml/min).

Rats blood sampling and treatment

Blood was collected from the tail vein of each rat during the inhalation exposure period at 1, 30, 60, 120, 180, 240, 300 and 360 min from the start of exposure. 0.1 ml blood samples were collected into 10-ml headspace sampler (HS)-vials and 0.1 ml of distilled water was added to each sample and the vials sealed immediately with an aluminum crimp cup.

Analysis of blood concentration

Blood samples were analyzed by HS-GC/mass spectrometry (MS) using Agilent Technologies 7694 (Agilent Technologies) headspace sampler (oven temperature, 80°C; loop temperature, 130°C; vial equilibration time, 10 min) and Agilent Technologies 5973N (Agilent Technologies) gas chromatography-mass spectrometry system (column, J&W DB-1 0.25 mmϕ × 60 m; oven temperature, 80°C; ion source temperature, 230°C; carrier gas, helium at 1 ml/min; ionization, EI; ionization voltage, 70 eV; fragment peak, 83 m/z).

RESULTS AND DISCUSSION

In the present study, a whole-body inhalation exposure system was designed to enable blood collection from rats during the period of exposure to a VOC; chloroform was used as a model VOC.

A performance study of the system without rats (study 1)

Table 1 (study 1) shows the chloroform vapor concentration at the sampling ports of the supply-header, animal-chamber and exhaust-header. The average concentrations in the supply-header, animal-chamber and exhaust-header over the course of a 6 hr exposure period were in the range of 100.0 to 101.5 ppm with coefficients of variation (C.V.) being less than 1.87%. The flow rate through each animal-chamber during this period was maintained within a range of 250 to 260 ml/min. Thus, the supply-header was able to supply uniform stable chloroform vapor to each animal-chamber, demonstrating good performance of the system.

Confirmation of system performance with rats (study 2)

Table 1 (study 2) shows the chloroform vapor concentration at the sampling ports of the supply-header, animal-chamber and exhaust-header. The average concentrations in the supply-header, animal-chamber and exhaust-head-