Table 5
Chlorate and perchlorate daily intake from 13 food groups.

Food groups		A*	B*	C	D	E	F	G*	H	I*	J
1) Chlorate (μg/day)											
1	Rice	0.1	2.7	9.6	30.0	24.0	22.0	8.5	32.0	2.1	9.6
2	Cereals and potatoes	4.4	19.0	10.0	14.0	15.0	8.3	5.1	7.2	3.2	11.0
3	Sugar and confectionaries	0.3	1.3	1.1	4.8	1.2	0.9	0.7	1.5	3.7	2.3
4	Oil and fats	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1
5	Legumes	5.2	7.5	6.1	5.4	4.7	5.8	12.0	15.0	2.4	16.0
6	Fruits	1.2	5.0	1.7	1.0	3.4	0.2	0.2	2.2	1.5	1.0
7	Green and yellow vegetables	0.3	0.4	2.0	4.0	3.0	1.2	1.2	2.6	0.6	1.3
8	Other vegetables and seaweed	2.1	1.7	4.4	6.3	14.0	11.0	1.9	5.6	5.7	3.9
9	Alcohols and beverages	45.0	9.9	16.0	33.0	30.0	27.0	14.0	81.0	5.3	50.0
10	Fish and shell fish	4.4	4.1	15.0	5.9	36.0	81.0	12.0	47.0	7.2	8.2
11	Meat and poultry	3.5	14.0	29.0	7.8	9.4	13.0	5.4	3.4	3.6	9.2
12	Milk products	6.4	13.0	4.7	9.6	17.0	10.0	4.5	14.0	8.7	8.7
13	Seasoning	6.2	5.0	2.8	4.7	3.5	7.8	1.7	2.7	3.6	5.2
14	Water	2260.0	117	90.4	1165.4	1159.0	2258.0	68.0	272.0	111.2	134.2
	Total	3339.2	2201.3	1192.8	2292.0	3320.2	4446.2	1135.2	486.2	158.8	260.6
2) Perchlorate (μg/day)											
1	Rice	0.064	0.45	0.17	0.70	0.16	0.075	0.16	0.09	0.018	0.036
2	Cereals and potatoes	0.41	4.8	1.3	1.9	2.5	0.46	1.3	0.07	0.24	0.93
3	Sugar and confectionaries	0.039	0.080	0.067	0.17	0.83	0.067	0.32	0.06	0.14	0.05
4	Oil and fats	0.002	0.003	0.002	0.001	0.004	0.001	0.003	0.0009	0.002	0.0027
5	Legumes	0.045	0.080	0.04	0.066	0.047	0.035	0.28	0.04	0.043	0.048
6	Fruits	0.17	0.057	1.0	0.33	0.084	0.22	0.05	0.06	0.043	0.067
7	Green and yellow vegetables	0.17	9.4	0.58	2.1	0.19	0.13	5.1	0.15	0.79	0.036
8	Other vegetables and seaweed	0.51	67	0.33	5.2	0.26	0.29	0.91	0.19	0.78	0.16
9	Alcohols and beverages	0.13	0.15	0.12	0.92	0.069	0.21	0.17	0.37	0.25	0.1
10	Fish and shell fish	0.14	0.11	0.26	1.9	0.21	0.14	0.24	0.21	0.11	1.1
11	Meat and poultry	0.45	0.13	0.16	0.37	0.063	0.13	0.17	0.1	0.048	0.15
12	Milk products	0.56	0.76	0.83	0.96	1.0	0.83	1.1	0.63	0.9	1.1
13	Seasoning	0.14	0.42	0.10	0.67	0.29	0.13	0.23	0.08	0.22	0.23
14	Water	0.10	0.42	0.32	4.40	0.26	0.36	0.06	0.46	0.26	0.24
	Total	2.9	83.9	5.3	19.7	6.0	3.1	10.1	2.5	3.8	4.2

* Pure water was used for food preparation all of which contained no chlorate (<0.05 μg/L) or perchlorate (<0.01 μg/L).

highest in VIII (1400 ng/g dry weight) followed by VI (810 ng/g dry weight). The daily intake was calculated by multiplying the measured concentration by the amount of formula per feeding, and the daily intake in 1 L of formula was calculated. The daily intake of chlorate was within the range of 8.4–196 μg/day for each sample. The measured concentration of perchlorate was high in I (22 ng/g dry weight) followed by II and VIII (each 10 ng/g dry weight). The daily intake of perchlorate was calculated in the same way as for chlorate, and ranged from 0.02 to 2.3 μg/day.

Using the measured concentration of TDS sample tap water to make the formula, the amounts of chlorate and perchlorate intake were as shown in Tables 6 and 7. The average daily intake of chlorate was 147 (42–332) μg/day for an ingestion volume of 1 L/day if prepared with tap water. In comparison to the TDI value of 150 μg/day for infants and toddlers calculated weighing 5 kg, some samples had higher daily intake levels.

The average daily intake of perchlorate was 1.1 μg/day ranged from 0.05 to 4.5 μg/day also for an ingestion volume of 1 L/day if prepared with tap water. There was no correlation between samples with high levels of chlorate intake and those with high perchlorate intake. The amounts were almost lower compared to the RfD value of 3.5 μg/day for infants and toddlers weighing 5 kg, but if tap water from location D with high intake was used to prepare the formula, total intake exceeded the RfD value (some samples were for toddlers weighing over 8.5 kg, and the daily intake also changes as infants grow), although it is sufficiently below PMTDI.

Using the combination of the mean, minimum and maximum concentration of chlorate and perchlorate in tap water examined in this study, the contribution of water for baby intake were calculated as 56% (15–94) for chlorate and 31% (1.3–99) for perchlorate. The range greatly depends upon the concentration of the sample baby formula and the concentration of water. It is difficult to conclude the contribution of water, because the number of sample baby formula and water was limited in this study, but the intake greatly depends on concentration of chlorate and perchlorate in water.

Table 6
Chlorate and perchlorate in baby formula.

		Intake (μg/1 L)	
		Chlorate	Perchlorate
I	WA	33.6	2.3
II	WA	15.6	0.98
III	MO	50.7	0.57
IV	MO	88.2	0.42
V	ME	63.8	0.04
VI	BS (liquid)	113.4	0.60
VII	ME	8.4	0.02
VIII	ME	196	1.05
	Av.	71.2	0.75

4. Discussion

The present study was performed using samples from the total diet study to evaluate the concentrations of chlorate and perchlorate

Table 7
Chlorate and perchlorate in baby formula.

	Chlorate intake (μg/day as 1 L)			Perchlorate intake (μg/day as 1 L)		
	Av.	Min.	Max.	Av.	Min.	Max.
Baby formula*	65	8	196	0.77	0.02	2.3
Water	82	34	136	0.34	0.03	2.2
Total	147	42	332	1.11	0.05	4.5

* Average is calculated excluding sample VI.

in food and tap water from 10 locations and calculate the total levels of intake. In most cases, the concentrations of chlorate and perchlorate from the various food groups were higher than the lower limit of quantification.

For the chlorate ion, the percentage contribution of tap water to total intake was as high as 47%–77%, and the level of intake in some food samples probably included chlorate originating from tap water. For example, chlorate in the tap water used for boiling and simmering of rice, potatoes, and noodles may have affected the amount of chlorate in food. The sum of the intakes from food and tap water for adults was less than approximately 32% of TDI.

A survey of the total intake of perchlorate and iodine was performed by the FDA in the USA, and the presence of perchlorate was confirmed in a wide variety of foods, as also demonstrated in the present intake survey (Murray et al., 2008). In the USA, the main sources of perchlorate intake were milk and dairy products, as well as vegetables. The intake from vegetables was relatively high in both countries. The survey in the USA classified the subjects into 14 categories according to age group and gender, and the amounts of intake for each category were compared. The intake of perchlorate for a 2-year-old was approximately 50% of the RfD value, and it seemed that younger age groups had higher intake ratios. As the study in the USA indicated that the average concentration of perchlorate in breast milk is 5 times higher than that in cow's milk (Kirk et al., 2005), it may be necessary to investigate the intake of perchlorate from breast milk in future studies.

In the present study, the measured concentration of perchlorate in dairy products was relatively high; however, when converted to the amount of intake, the levels were not high. Vegetables, seaweed, cereals and potatoes showed specifically high perchlorate levels at certain locations. Location D, which had high levels of perchlorate intake from tap water, had relatively high total intake corresponding to 56% of the RfD, and this was considerably higher than at the other locations. There was no obvious relationship whether tap water was used to prepare food, but further studies are required regarding the concentrations in both tap water and food from the viewpoint of total intake.

The perchlorate intake from "other vegetables and seaweed" at location B was relatively high. Because the food samples were analyzed in composite for each group, this study was not able to further reveal which individual food(s) were actually of high perchlorate concentration. But it should be noted seaweed, which is generally known to contain high perchlorate and iodine (Martinelango et al., 2006, for example), was included in location B and other locations. Japanese diet normally includes a high proportion of seafood especially seaweeds, such as Wakame (*Undaria pinnatifida*). Although it is known that perchlorate interferes with iodine uptake, adverse health effect from iodine deficiency might not be occurred if iodine intake from foods is high and if perchlorate intake is relatively low. Because iodine uptake from Japanese normal diet is relatively higher than other countries (Kikuchi et al., 2008), so evaluation of both perchlorate and iodine intake should be conducted in future when considering perchlorate and iodine uptake.

To our knowledge, no detailed data are available regarding water consumption for drinking, cooking use, both only boiling and making soup, ice, and other uses for ingestion. Therefore, we are currently engaged in a national survey of water consumption.

The results of the present study revealed the total intake, as well as the percentage intake from water, of chlorate and perchlorate in Japan. With the studied areas being selected from across Japan, the percentage contribution of chlorate and perchlorate from water to the total amount of intake was first proved. The contribution of water used for preparation of food was also found as an important factor in the present study. The contribution of total daily intake of chlorate originated from water including water used for preparation of food in this study was lower than the current TDI allocation of chlorate to water (i.e., 80%) and also the total intake of chlorate was much the less than the TDI. The contribution of perchlorate and total perchlorate intake ranged widely, therefore it needs further studies when considering the current allocation (i.e., 10%).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.05.097.

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Relative source allocation of TDI to drinking water for derivation of a criterion for chloroform: A Monte-Carlo and multi-exposure assessment

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ABSTRACT

Drinking water quality standard (DWQS) criteria for chemicals for which there is a threshold for toxicity are derived by allocating a fraction of tolerable daily intake (TDI) to exposure from drinking water. We conducted physiologically based pharmacokinetic model simulations for chloroform and have proposed an equation for total oral-equivalent potential intake via three routes (oral ingestion, inhalation, and dermal exposures), the biologically effective doses of which were converted to oral-equivalent potential intakes. The probability distributions of total oral-equivalent potential intake in Japanese people were estimated by Monte Carlo simulations. Even when the chloroform concentration in drinking water equaled the current DWQS criterion, there was sufficient margin between the intake and the TDI: the probability that the intake exceeded TDI was below 0.1%. If a criterion that the 95th percentile estimate equals the TDI is regarded as both providing protection to highly exposed persons and leaving a reasonable margin of exposure relative to the TDI, then the chloroform drinking water criterion could be a concentration of 0.11 mg/L. This implies a daily intake equal to 34% of the TDI allocated to the oral intake (2 L/d) of drinking water for typical adults. For the highly exposed persons, inhalation exposure via evaporation from water contributed 53% of the total intake, whereas dermal absorption contributed only 3%.
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1. Introduction

1.1. Background

Drinking water quality guideline values of threshold chemicals, which have been assumed to have safe exposure levels up to a certain threshold level, are derived by allocating a fraction of tolerable daily intake (TDI) to exposure from drinking water. This allocation is made because drinking water is not the sole source of chemical exposure, and it is essential to take into account exposure from other routes. The World Health Organization's Guidelines for Drinking-water Quality say, "Some consideration of the proportion of the ADI [acceptable daily intake] or TDI that may be attributed to different sources is therefore needed in developing guideline values" (WHO, 2011). Where sufficient information on exposure is available, the amount of intake that comes from drinking water and its relative contribution to total exposure can be estimated. However, the calculation of the allocation factor, the fraction of the TDI allocated to drinking water, is not clearly defined.

USEPA (2000) recommends the Exposure Decision Tree Approach for TDI (termed the reference dose [RfD]) allocation. The approach utilizes either the subtraction or percentage method to account for other exposures, depending on whether one or more health-based criterion is relevant for a chemical in question. The subtraction method is considered acceptable when only one criterion is relevant for a chemical, while the percentage method is recommended when multiple media criteria are under consideration. The USEPA says "The subtraction method results in a criterion allowing the maximum possible chemical concentration in water after subtracting other sources. As such, it removes any cushion between pre-criteria levels (i.e., actual "current" levels) and the RfD". In this approach, however, the drinking water intake estimate is approximately the 90th percentile value, whereas intake estimates from non-water exposures are based on arithmetic mean values. EPA says that this combination of parameter value assumptions is expected to result in a criterion that is protective of a majority of the population, but does not recommend that high-end intakes

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Abbreviations

Symbols	Definition
a_i	coefficient for the effect of cooking water on food (dimensionless)
A_D	daily oral intake (mg/day)
A_f	allocation factor (%)
A_{sk}	body surface area (cm^2)
$b_k(r)$	coefficient for the effect of water evaporation on the air (dimensionless, k=bathroom, kitchen, or residence)
b_w	body weight (kg)
b_h	body height (cm)
C_a	concentration in inhaled air (mg/L)
$\overline{C}_a(r)$	daily-average concentration in inhaled air (mg/L)
$C_{air,w,k}$ (r)	concentration in inhaled air in an area (k = bathroom, kitchen, or residence) under the influence from tap water (mg/L) ("(r)" indicates Monte-Carlo input.)
$C_{air,outdoor}$ (r)	concentration in outdoor air (mg/L)
C_{alv}	concentration in alveolar air (mg/L)
C_{art}	concentration in arterial blood (mg/L)
C_d	concentration in water for dermal adsorption (mg/L)
$\overline{C}_d(r)$	daily-average concentration in water for dermal adsorption (mg/L) ("(r)" indicates Monte-Carlo input.)
$C_{food,w,i}$	concentration in ith food group after cooking with tap water (mg/g)
$C_{food,0,i}$	concentration in ith food group after cooking with pure water (mg/g)
C_{ven}	concentration in mixed venous blood (mg/L)
C_{vf}	concentration in venous blood leaving fat (mg/L)
C_{vk}	concentration in venous blood leaving the kidneys (mg/L)
C_{vl}	concentration in venous blood leaving the liver (mg/L)
C_{vr}	concentration in venous blood leaving rapidly perfused tissues (mg/L)
C_{vs}	concentration in venous blood leaving slowly perfused tissues (mg/L)
C_{vsk}	concentration in venous blood leaving skin (mg/L)
C_w	concentration in tap water (mg/L)
D_D	dermal potential dose [mg/(kg-body d)]
D_{DO}	oral-equivalent dermal potential dose [mg/(kg-body d)]
D_I	inhalation potential dose [mg/(kg-body d)]
D_{IO}	oral-equivalent inhalation potential dose [mg/(kg-body d)]
D_O	oral potential dose [mg/(kg-body d)]
D_T	total oral-equivalent potential dose [mg/(kg-body d)]
E_D	dermal biologically effective dose [mg/(kg-organ d)]
E_I	inhalation biologically effective dose [mg/(kg-organ d)]
E_O	oral biologically effective dose [mg/(kg-organ d)]
G_v	guideline value (mg/L)
K_{mk}	Michaelis constant for enzymatic reaction for the kidneys (mg/L)
K_{ml}	Michaelis constant for enzymatic reaction for the liver (mg/L)
K_p	effective skin permeability coefficient (cm/d)
$I_{food,i,j}$ (r)	daily intake of jth food in ith food group (g/d) ("(r)" indicates Monte-Carlo input.)
I_{water}	daily drinking water consumption (L/d)
P_{ba}	blood/air partition coefficient (dimensionless)
P_{fb}	fat/blood partition coefficient (dimensionless)
P_{kb}	kidney/blood partition coefficient (dimensionless)
P_{lb}	liver/blood partition coefficient (dimensionless)
P_{rb}	rapidly perfused/blood partition coefficient (dimensionless)
P_{sb}	slowly perfused/blood partition coefficient (dimensionless)
P_{skb}	skin/blood partition coefficient (dimensionless)
P_{skw}	skin/water partition coefficient (dimensionless)
Q	breathing rate (L/d)
Q_{alv}	alveolar ventilation rate (L/d)
Q_f	blood flow rate to fat (L/d)
Q_k	blood flow rate to the kidneys (L/d)
Q_l	blood flow rate to the liver (L/d)
Q_r	blood flow rate to rapidly perfused tissues (L/d)
Q_s	blood flow rate to slowly perfused tissues (L/d)
Q_{sk}	blood flow rate to skin (L/d)
Q_t	cardiac output (L/d)
t	time (d)
$t_{bathroom}$(r)	time spent in bathroom per day (dimensionless)
$t_{kitchen}$(r)	time spent in kitchen per day (dimensionless)
$t_{residence}$(r)	time spent in residence per day (dimensionless)
$t_{outdoor}$(r)	time spent outdoors per day (dimensionless)
V_f	volume of fat (L)
V_l	volume of the liver (L)
V_k	volume of the kidneys (L)
V_{maxl}	maximum enzymatic reaction rate for the liver (mg/d)
V_{maxk}	maximum enzymatic reaction rate for the kidneys (mg/d)
V_r	volume of rapidly perfused tissues (L)
V_s	volume of slowly perfused tissues (L)
V_{sk}	volume of skin (L)
α	ratio of effective doses by single/continuous exposure (dimensionless)
α_1	ratio of oral effective doses by single/continuous exposure (dimensionless)
α_2	ratio of inhalation effective doses by single/continuous exposure (dimensionless)
α_3	ratio of dermal effective doses by single/continuous exposure (dimensionless)
$\alpha_{2/1}$	ratio of α_2 to α_1 (dimensionless)
$\alpha_{3/1}$	ratio of α_3 to α_1 (dimensionless)
β	ratio of effective/potential dose at a constant continuous administration (kg-body d/kg-organ)
β_1	ratio of oral effective/potential dose at a constant continuous administration (kg-body d/kg-organ)
β_2	ratio of inhalation effective/potential dose at a constant continuous administration (kg-body d/kg-organ)
β_3	ratio of dermal effective/potential dose at a constant continuous administration (kg-body d/kg-organ)
$\beta_{2/1}$	ratio of β_2 to β_1 (dimensionless)
$\beta_{3/1}$	ratio of β_3 to β_1 (dimensionless)
ϕ	ratio of alveolar ventilation rate to breathing rate (dimensionless)

be subtracted for every exposure source since the combination may not be representative of any actually exposed population.

For volatile compounds such as chloroform and benzene, volatilization from water, in particular when bathing, may increase exposure via inhalation, which could raise total exposure. Dermal absorption of compounds when bathing could also raise total exposure. Shehata (1985) performed a multi-route exposure assessment for benzene, toluene, and xylene. The study employs the subtraction method and suggests that 0–64% (average: 32%) of exposure can be allocated to drinking water. For trihalomethanes (THMs), exposure through inhalation while showering is estimated to be 50–200% of oral exposure via ingestion of water (Jo et al., 2005; Kim et al., 2004). The ratios of dermal exposure to oral exposure are estimated to be 30–70% for THMs (Xu et al., 2002). Ingestion of drinking water is estimated to account for 4–24% of exposure for THMs, whereas combined exposure from inhalation

and dermal absorption accounts for 69–94% of total exposure (Yanagibashi et al., 2010). These previous studies have estimated the contributions to be the ratios to total potential exposure (intake of administered dose) or total exposure on the assumption that absorption is constant. Jo et al. (1990) measured chloroform concentrations in exhaled breath at a certain time after exposure. The concentration in exhaled breath may be a more appropriate index to evaluate internal exposure than potential dose, but whether it represents the chemical burden in the target organ is uncertain. Chemicals may enter the bloodstream at different rates depending on the exposure route (oral, inhalation, and dermal) and may also be metabolized differently. The net effect of volatilization and dermal absorption upon total exposure should therefore be evaluated based on changes of the chemical burden in the target organ, for example, liver and kidney (Lévesque et al., 2000).

Although there has been an accumulation of scientific knowledge relevant to assessment of exposure to various chemical compounds, two issues remain to be investigated. The first issue is determination of the total multi-route exposure of a target organ by consideration of the differences between potential dose and biologically effective dose for each exposure route. Addressing this issue will require more rigorous multi-route exposure assessments based on route-to-route extrapolation (IGHRC, 2006). The second issue is quantitative evaluation of the cushion or margin between actual exposure levels and the TDI needed to protect the majority of the population. Risk analyses are needed for determining criteria that provide protection to, for example, 95% of the population or the highly exposed population and/or that leave reasonable margins of exposure relative to TDIs.

1.2. Objectives

The objective of the present study was to propose, based on an assessment of exposure and risk, a method for evaluating the allocation factor to drinking water needed to establish the drinking water quality standard (DWQS) for a chemical of interest. We chose chloroform as the target chemical for which there is a threshold for toxicity. Chloroform is a carcinogen but it has been concluded that there is a threshold mechanism for carcinogenicity because the carcinogenic activity of chloroform is mediated by a non-genotoxic mechanism of action that is secondary to cytotoxicity and cellular proliferation (USEPA, 2001; WHO, 2005b). Therefore, the cancer risk for chloroform is assessed based on TDI, rather than the linear low-dose non-threshold/slope factor approach.

We conducted physiologically based pharmacokinetic (PBPK) model simulations and have proposed the concept of a total oral-equivalent potential dose from multi-route exposure. Next, we used Monte Carlo simulations to estimate the distribution of total oral-equivalent potential doses of chloroform resulting from variability of exposures in a population. We assumed a 2-L/day water ingestion rate and a 50-kg body weight; these are the assumptions used in the DWQS determinations in Japan (MHLWJ, 2003). We then calculated the allocation factor to drinking water for chloroform based on the Monte-Carlo and multi-exposure assessments.

2. Methods

2.1. Total oral-equivalent potential dose

Although TDI is determined from administered doses, the toxicity of a compound is determined by the biologically effective dose, which is the dose to which the organ of interest is exposed. The biologically effective dose is the portion of the potential dose (administered dose) that reaches the site of toxic action, and it is proportional to the administered dose. Therefore, we define β as the ratio of effective/potential dose at a constant rate of continuous administration. When the level of exposure varies with time, the time-averaged daily dose is a useful metric, but the effect may depend on the temporal variation of the level of exposure. The variation of exposure may result in a lower effective dose than a constant continuous exposure, even though the time-averaged potential doses are the same. We thus define α to be the ratio of effective doses from single and continuous exposures. When average potential doses from oral ingestion, inhalation, and dermal exposure are given by Eqs. (1), (2), 3) in Table 1, respectively, their biologically effective doses are given by Eqs. (4), (5), (6), respectively, by introducing α and β. A dose associated with inhalation or dermal exposure may be converted into a hypothetical potential dose from oral ingestion such as the equivalent biologically effective dose. In this study, we designate the converted hypothetical potential doses associated with inhalation and dermal exposure as oral-equivalent potential doses from inhalation or dermal exposure, respectively. For example, when the oral-equivalent potential dose of inhalation is 1 mg/(kg d), that dose produces the same biologically effective dose as an oral potential dose of 1 mg/(kg d). Oral-equivalent potential doses from inhalation and dermal exposure are given by Eqs. (7) and (8), respectively. Finally, the total oral-equivalent potential dose via three routes (oral ingestion, inhalation, and dermal exposures) can be calculated from:

$$D_T = \frac{A_D}{b_w} + \alpha_{2/1}\beta_{2/1}\frac{\overline{C_a}\phi Q}{b_w} + \alpha_{3/1}\beta_{3/1}\frac{K_p A_{sk}\,\overline{C_d}}{b_w \times 1000\ \mathrm{cm^3/L}} \tag{9}$$

The value of $\beta_{2/1}$ can be calculated by combining Eqs. (1), (2), (4), and (5):

$$\beta_{2/1} \equiv \frac{\beta_2}{\beta_1} = \frac{\alpha_1 D_O}{\alpha_2 C_a \phi Q / b_w} \times \frac{E_I}{E_O} \tag{10}$$

When the biologically effective doses for oral and inhalation exposures are the same ($E_O = E_I$) under conditions of constant continuous exposure ($\alpha_1 = \alpha_2 = 1$),,

$$\beta_{2/1} = \frac{b_w D_O}{Q_{alv} C_a} \tag{11}$$

A similar procedure can be used for $\beta_{3/1}$. When $E_O = E_D$, $\beta_{3/1}$ is given by Eq. (12):

$$\beta_{3/1} \equiv \frac{\beta_3}{\beta_1} = \frac{D_O}{D_D} = \frac{D_O}{K_p A_{sk} C_d / (b_w \times 1000\ \mathrm{cm^3/L})} \times \frac{E_D}{E_O} = \frac{b_w \times 1000\ \mathrm{cm^3/L}}{K_p A_{sk}} \times \frac{D_O}{C_d} \tag{12}$$

When chloroform is inhaled at a constant rate with no variation in concentration, the chloroform concentration in the venous blood reaches steady state. For intermittent dose with an interval at steady state, effects resulting from subchronic or chronic exposure would normally be related to the area under the curve (AUC), whereas acute toxicity for a single exposure could be related to either the AUC or the peak concentration (WHO, 2005a). The AUC after administration of a single dose extrapolated to infinity is a suitable alternative to the AUC for an intermittent dose at steady state. Accordingly, the average chloroform concentration associated with a single exposure is given by the AUC divided by the duration of exposure. Therefore, the ratio of effective doses for intermittent and constant-rate exposure is given by:

$$\alpha_i = \frac{\mathrm{AUC/exposure\ time}}{\mathrm{steady\ state\ concentration}}; \quad i = 1 \text{ to } 3 \tag{13}$$

2.2. PBPK model

The PBPK model can be used to quantify the kinetic behavior (absorption, distribution, metabolism, and excretion) in the body

Table 1
Exposures and doses.

Exposure	Potential dose		Biologically effective dose		Oral-equivalent potential dose	
Oral						
	$D_O = \frac{A_D}{b_w}$	(1)	$E_O = \alpha_1 \beta_1 D_O$	(4)	$D_O = \frac{A_D}{b_w}$	(1)
Inhalation						
	$D_I = \frac{\overline{C_a} Q}{b_w}$	(2)	$E_O = \alpha_2 \beta_2 \phi D_I$	(5)	$D_{IO} = \alpha_{2/1} \beta_{2/1} \phi D_I$	(7)
Dermal						
	$D_D = \frac{K_p A_{sk} \overline{C_d}}{(1000 cm^3/L) b_w}$	(3)	$E_O = \alpha_3 \beta_3 D_D$	(6)	$D_{DO} = \alpha_{3/1} \beta_{3/1} D_D$	(8)

of chemicals absorbed via multiple exposure routes (IPCS, 2010). The model can predict the concentration of the toxic substance in the target organ, which is relevant to the effect of the toxin, and are used for the prediction of route-to-route extrapolation for exposure assessment and the prediction of inter-individual and interspecies differences in dose/concentration–response assessment (IPCS, 2010). In the present study, we used the PBPK model developed for chloroform by Tan et al. (2006) to enable route-to-route extrapolation. The model, whose structure is depicted in Fig. 1, is expressed mathematically by a set of simultaneous differential equations that quantify the rate of change of chloroform concentration in each compartment. We used the following equations in the present study (Corley et al., 2000, 1990; Ramsey and Andersen, 1984; Tan et al., 2006).

The equations for the compartments associated with rapidly perfused tissues, slowly perfused tissues, and fat were respectively as follows:

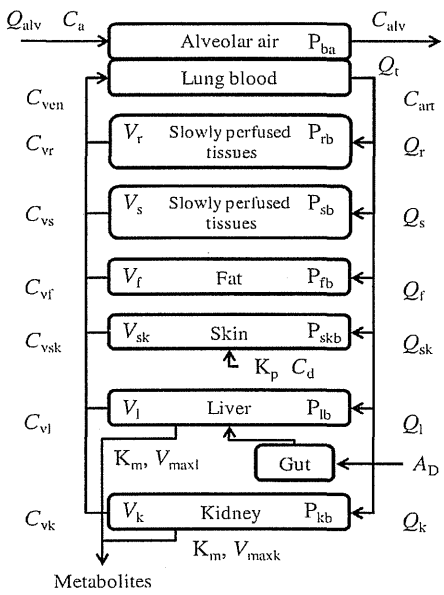

Fig. 1. Diagram of PBPK model used to describe the disposition of chloroform in humans via oral ingestion, inhalation, and dermal exposure.

$$V_r P_{rb} \frac{dC_{vr}}{dt} = Q_r (C_{art} - C_{vr}) \quad (14)$$

$$V_s P_{sb} \frac{dC_{vs}}{dt} = Q_s (C_{art} - C_{vs}) \quad (15)$$

$$V_f P_{fb} \frac{dC_{vf}}{dt} = Q_f (C_{art} - C_{vf}) \quad (16)$$

The skin compartment was described by the following equation:

$$V_{sk} P_{skb} \frac{dC_{vsk}}{dt} = Q_{sk} (C_{art} - C_{vsk}) + \frac{K_p A_{sk}}{1000} \left(C_d - \frac{P_{skb}}{P_{skw}} C_{vsk} \right) \quad (17)$$

The equations for the compartments associated with the liver and kidneys took into consideration the metabolism of those organs. Those equations were as follows:

$$V_l P_{lb} \frac{dC_{vl}}{dt} = Q_l (C_{art} - C_{vl}) - \frac{V_{maxl} C_{vl}}{K_{ml} + C_{vl}} + A_D \quad (18)$$

$$V_k P_{kb} \frac{dC_{vk}}{dt} = Q_k (C_{art} - C_{vk}) - \frac{V_{maxk} C_{vk}}{K_{mk} + C_{vk}} \quad (19)$$

Eq. (20) was used to calculate arterial chloroform concentrations in the simulations.

$$C_{art} = \frac{Q_{alv} C_a + Q_t C_{ven}}{Q_t + Q_{alv}/P_{ba}} \quad (20)$$

The concentration of chloroform in mixed venous blood returning to the lung was calculated from the sum of the contributions of venous blood from each of the six tissue groups as indicated by Eq. (21):

$$C_{ven} Q_t = Q_r C_{vr} + Q_s C_{vs} + Q_f C_{vf} + Q_{sk} C_{vsk} + Q_l C_{vl} + Q_k C_{vk} \quad (21)$$

We used Mathematica 9 (Wolfram Research, Champaign, IL, USA) to solve the equations as a system of ordinary differential equations. We conducted three kinds of simulations by solving the equations with three datasets [designated as PBPK Model Parameter Sets (PMPSs) A, B, and C] of model input parameter values (Table 2). We obtained three PMPSs from the literature (Corley et al., 2000, 1990; Environment Canada and Health Canada, 2001; Tan et al., 2006). The changes of chloroform concentrations in each compartment were quantified. Among the model parameters, inhalation and dermal exposure rates were most influenced by and sensitive to the alveolar ventilation rate and the effective skin

Table 2
Parameter values for the PBPK model.

		Parameters		PBPK model parameter set (PMPS)		
				A	B	C
Body weight		b_w	(kg)	70[b]	70[b]	79.9[c]
Tissue volume (assume unit density)[a]	Rapidly perfused	V_r	(L)	3.77[a]	4.96[b]	2.61[c]
	Slowly perfused	V_s	(L)	39.3[a]	30.2[b]	44.7[c]
	Fat	V_f	(L)	15.0[a]	15.0[b]	18.5[c]
	Skin	V_{sk}	(L)	3.57[a]	3.57[c]	4.07[c]
	Liver	V_l	(L)	1.80[a]	1.80[b]	2.51[c]
	Kidney	V_k	(L)	0.308[a]	0.308[b]	0.352[c]
Alveolar ventilation rate		Q_{alv}	(L/d)	13940[a]	13940[b]	9208[c]
Breathing rate		Q	(L/d)	19110[d]	19110[d]	19110[d]
Ratio of alveolar ventilation rate to breathing rate		ϕ	(dimensionless)	0.729	0.729	0.482
Cardiac output		Q_t	(L/d)	9583[a]	9583[b]	9208[c]
Blood flow	Rapidly perfused	Q_r	(L/d)	2434[a]	1054[c]	460[c]
	Slowly perfused	Q_s	(L/d)	1629[a]	2453[b]	1473[c]
	Fat	Q_f	(L/d)	479[a]	498[b]	1013[c]
	Skin	Q_{sk}	(L/d)	824[a]	1725[c]	1658[c]
	Liver	Q_l	(L/d)	2396[a]	2175[b]	2302[c]
	Kidney	Q_k	(L/d)	1821[a]	1677[b]	2302[c]
Partition coefficients	Blood/air	P_{ba}	(dimensionless)	7.43[a]	7.43[b]	7.43[c]
	Rapidly perfused/blood	P_{rb}	(dimensionless)	2.29[a]	2.29[b]	2.29[c]
	Slowly perfused/blood	P_{sb}	(dimensionless)	1.62[a]	1.62[b]	1.62[c]
	Fat/blood	P_{fb}	(dimensionless)	37.69[a]	37.69[b]	37.69[c]
	Skin/blood	P_{skb}	(dimensionless)	1.62[a]	1.62[c]	1.62[c]
	Skin/water	P_{skw}	(dimensionless)	3.85[a]	3.85[c]	3.85[c]
	Liver/blood	P_{lb}	(dimensionless)	2.29[a]	2.29[b]	2.29[c]
	Kidney/blood	P_{kb}	(dimensionless)	1.48[a]	1.48[b]	1.48[c]
Maximum reaction rate	Liver	V_{maxl}	(mg/d)	7374[a]	26380[b]	30110[c]
	Kidney	V_{maxk}	(mg/d)	41.79[a]	149.5[b]	139.6[c]
Michaelis constant	Liver	K_{ml}	(mg/L)	0.448[a]	0.448[b]	0.448[c]
	Kidney	K_{mk}	(mg/L)	0.448[a]	0.448[b]	0.448[c]
Effective skin permeability coefficient		K_p	(cm/d)	1.2[a]	1.416[c]	1.416[c]
Body surface area exposed		A_{sk}	(cm^2)	20020[a]	20020[a]	21951[c]

[a] Tan et al. (2006).
[b] Environment Canada and Health Canada (2001).
[c] Corley et al., 1990; Corley et al. (2000).
[d] USEPA (2011).

permeability coefficient. We used the experimental data of Jo et al. (1990) and Nuckols et al. (2005) to verify these parameter values (see S.1 of Supplementary Information).

2.3. Monte-Carlo and multi-exposure assessment

We calculated the probability distribution functions for total oral-equivalent exposure to chloroform via the three routes (ingestion via drinking water and food, inhalation, and dermal absorption) by using Eq. (9) for a given chloroform concentration in drinking water and Monte Carlo inputs (Crystal Ball 2000, Japanese edition, Kozo Keikaku Engineering Inc., Tokyo, Japan) with 10^5 trials and a random number sampling method (Latin Hypercube Sampling). Monte Carlo inputs were those related to the characterization of the exposure scenarios, i.e., rates of food intake, chloroform concentrations in the air, inhalation exposure times, and dermal exposure times. However, consumption of drinking water and the body weight were set to 2 L/day and 50 kg, respectively, according to the assumptions employed in the DWQS determination in Japan, as described previously. Body height was assumed to be 160 cm, which represents the Japanese adult population (MHLWJ, 2006). Table 3 shows the assumed alveolar ventilation rate and exposed body surface area for a person weighing 50 kg. Likewise, physiological model parameters (alveolar ventilation rate, exposed body surface area, $\alpha_{2/1}$, $\alpha_{3/1}$, $\beta_{2/1}$, and $\beta_{3/1}$) were deterministic. Monte Carlo inputs were prepared based on data for ordinary Japanese adults; therefore, the distributions of total oral-equivalent exposure generated by the simulations do not reflect children and special high-exposure groups, such as people working in high-exposure occupations.

2.3.1. Procedures for estimating exposures via each route

2.3.1.1. Oral exposure. Ingestion of drinking water and food was considered to be exposure from oral intake. Foods were categorized into 17 groups. Chloroform concentrations in a composite of each group have been reported (Itoh and Asami, 2010). We used Eq. (22) to also take into account the indirect ingestion of chloroform in tap water through cooking because chloroform in tap water affects the chloroform concentration in cooked food as a result of the cooking process (Itoh and Asami, 2010).

$$C_{\text{food,w,i}} = a_i \times C_w + C_{\text{food,0,i}} \tag{22}$$

To calculate the exposure to chloroform via food, the chloroform concentration in each food composite group was multiplied by the daily intake of that food group. The exposure to chloroform from dietary consumption was calculated by adding the daily chloroform intake from all food groups. Daily intake of each food group was determined randomly by the Monte Carlo method based on the probability distributions of the data from the National Health and Nutrition Survey (Fig. 3S, Supplementary Information) (MHLWJ, 2006). Intakes of foods were assumed to be independent each other, with the exception of staples and portentous foods, where very weak correlations were introduced between rice and bread consumption, and among the intakes of pork, eggs, fish, poultry, milk, and beef (see Supplementary Information). Finally, daily oral intake of chloroform was estimated from Eq. (23).

$$A_D = C_w I_{\text{water}} + \sum_{i=1}^{17}\left[C_{\text{food,w,i}} \sum_j I_{\text{food,i,j}}(r)\right] \tag{23}$$

Table 3
Values of alveolar ventilation rate, body surface area exposed, $\alpha_{2/1}$, $\beta_{2/1}$, $\alpha_{3/1}$, and $\beta_{3/1}$ used for Monte-Carlo simulations. The body weight and height are assumed to represent the Japanese adult population (MHLWJ, 2006). Alveolar ventilation rate and exposed body surface area in Exposure Parameter Sets (EPS) A, B, and C were calculated with the same equations used for PMPSs A, B, and C, respectively. The values of $\alpha_{2/1}$, $\alpha_{3/1}$, $\beta_{2/1}$, and $\beta_{3/1}$ in EPSs A, B, and C were calculated with PMPSs A, B, and C, respectively.

Parameters			Exposure parameter set (EPS)		
			A	B	C
Body weight	b_w	(kg)	50	50	50
Body height	b_h	(cm)	Not used	Not used	160
Alveolar ventilation rate	Q_{alv}	(L/d)	10830	10830	6509
Body surface area exposed	A_{sk}	(cm^2)	14300	14300	16550
Ratio of α_2 to α_1	$\alpha_{2/1}$	(dimensionless)	1	1	1
Ratio of α_3 to α_1	$\alpha_{3/1}$	(dimensionless)	1	1	1
Ratio of β_2 to β_1	$\beta_{2/1}$	(dimensionless)	0.550	0.502	0.603
Ratio of β_3 to β_1	$\beta_{3/1}$	(dimensionless)	0.542	0.498	0.597

Eq. (23) was substituted into Eq. (9) to calculate oral dosage, but this substitution implicitly ignores any difference in gastrointestinal absorption rates between food products. We had no other choice than to assume a constant absorption rate, regardless of food products, because no relevant information was available. Chloroform concentrations in foods were not simulated with Monte Carlo methods because no relevant data were available. However, this omission should be of little consequence because the oral intake from food was not a major contributor (vide infra).

2.3.1.2. Exposure from inhalation. Ambient air associated with exposure from inhalation was classified into air in four location categories: bathroom for bathing and showering; kitchen; other indoor areas in a residence; and other areas, including outdoors and work places (hereafter simply referred to as outdoors). Chloroform concentrations in the former three areas were assumed to be affected by the volatilization of chloroform in tap water. Although the magnitude of their effects are dependent on many factors (ventilation, purpose of water use, quantity of water consumed, etc.), chloroform concentrations can basically be expressed by Eq. (24).

$$C_{air,w,i}(r) = b_k(r) \times C_w + C_{air,outdoor}(r) \tag{24}$$

The chloroform concentrations in outdoor air were obtained from Itoh and Asami (2010) and used as Monte-Carlo input (Fig. 4S, Supplementary Information). The parameter b_k(r) was determined for each residence in the data (Itoh and Asami, 2010) with the use of Eq. (24). The obtained distribution of b_k(r) values was used for the Monte-Carlo input (Fig. 5S, Supplementary Information).

The amount of time spent at each location was obtained from three studies. The probability distribution of the amount of time spent per day bathing and showering in the bathroom was obtained from (ULRI, 1999) (Fig. 6S, Supplementary Information). The data for the amount of time spent in the kitchen was obtained from the survey by Itoh and Asami (2010) (Fig. 6S, Supplementary Information). NHK-BCRI (2006) reported the amount of time spent in the residence (Fig. 6S, Supplementary Information). These data were used for the Monte-Carlo inputs. Once the amount of time spent per day in the bathroom, kitchen, and residence were determined in the Monte-Carlo input, the amount of time spent in the residence, except for the bathroom and kitchen (hereafter simply referred to as residence) was determined by subtraction, and the amount of time per day spent outdoors was also determined by subtraction.

We used Eq. (25) to estimate daily average chloroform concentrations in inhaled air, given a set of concentrations and amounts of time spent in the four areas in the Monte-Carlo input.

$$\begin{aligned}\overline{C_a}(r) = {} & C_{air,w,bathroom}(r)t_{bathroom}(r) + C_{air,w,kitchen}(r)t_{kitchen}(r) \\ & + C_{air,w,regidence}(r)t_{regidence}(r) + C_{air,outdoor}(r)t_{outdoor}(r)\end{aligned} \tag{25}$$

2.3.1.3. Dermal exposure. Dermal exposure was assumed to occur only in the bathroom during bathing and showering (dermal exposure from other activities, such as swimming, was neglected). It was assumed that the entire surface of the body was in contact with tap water during bathing and showering, and the values for body surface area were calculated by assuming a body weight of 50 kg and the equations applied in previous studies (Corley et al., 2000; Tan et al., 2006). The probability distribution of the amount of time spent bathing and showering in the bathroom was obtained from ULRI (1999), as explained before. Finally, we used Eq. (26) to calculate the daily average concentration for dermal exposure.

$$\overline{C_d}(r) = C_w t_{bathroom}(r) \tag{26}$$

3. Results and discussion

3.1. Equation to estimate total oral-equivalent potential dose

3.1.1. Estimations of $\beta_{2/1}$ and $\beta_{3/1}$

The TDI of chloroform is determined from administered doses based on the incidence of hepatic cysts (MHLWJ, 2003). Chloroform induces hepatic toxic effect via production of its reactive metabolites in liver. Relationship between the biologically effective dose and the hepatic toxicity are submitted into the scheme of pharmacodynamics, where a mechanism for the induction of tumours following hepatic exposure to chloroform is represented by cytotoxicity, which is primarily related to rates of chloroform metabolisms (Lévesque et al., 2000, 2002; Liao et al., 2007; Reitz et al., 1990). Therefore, the rate of chloroform metabolism generally has been identified as the preferred dose surrogate for correlating pharmacokinetics with the adverse hepatic effects of chloroform (Sasso et al., 2013; Tan et al., 2003). We calculated chloroform metabolism rates for several oral doses [0.0015–0.15 mg/(kg d)], inhalation concentrations (0.014–2.2 mg/m^3), and dermal exposure concentrations (0.006–0.7 mg/L). The chloroform metabolism rates were linearly increased with does and with exposure concentrations (Fig. 7S, Supplementary Information). The relationships between oral dose and inhalation concentration at equal chloroform metabolism rates (the lines in Fig. 2) were obtained from the results [Fig. 7S (A) and (B)]. The same relationships were obtained when hepatic tissue dose instead of metabolism rates was used as dose surrogate (data not shown). As shown in Fig. 2, the plot of both TDI vs. RfC (reference concentration) for the incidence of hepatic cysts reported by Environment Canada and Health Canada (2001) was near the lines obtained with the PBPK model simulation with PMPSs A and B, which verified the relationships between oral dose (D_O) and inhalation concentration (C_a) obtained by our model calculations. Substituting the slope (D_O/C_a) into Eq. (11) yielded the $\beta_{2/1}$ values (Table 3). The $\beta_{2/1}$

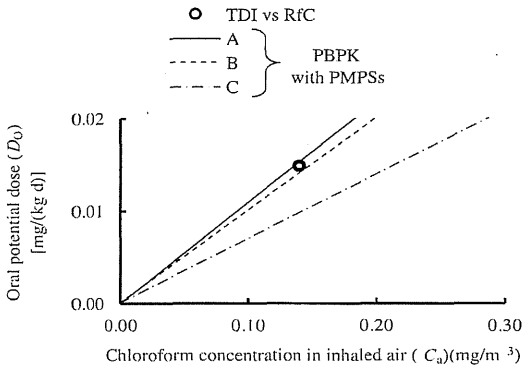

Fig. 2. Relationship between oral potential dose of chloroform (D_O) and chloroform concentration in inhaled air (C_a). Lines indicate the relationship that gave at equal chloroform metabolism rates with the PBPK model simulations and with PMPSs A, B, and C. The point indicates the relationship between TDI and RfC reported by Environment Canada and Health Canada (2001).

values were in the range of 0.502–0.603. Chiu and White (2006) propose a steady-state solution for the ratio of the inhaled alveolar dose to the oral dose that gives the equivalent internal dose at liver. The ratio in their study is regarded as the reciprocal of $\beta_{2/1}$:

$$\frac{1}{\beta_{2/1}} = \frac{Q_{alv}C_a}{b_w D_O} = 1 + \frac{Q_{alv}/P_{ba}}{Q_l} \tag{27}$$

The $\beta_{2/1}$ values calculated by their equation [Eq. (27)] with the parameter values of Table 2 were in the range of 0.56–0.65, which were roughly consistent with our evaluation (Table 3).

Fig. 3 shows the relationship between oral doses and dermal exposure concentrations that yielded equal chloroform metabolism rates [Fig. 7S (A) and (C), Supplementary Information]. Substituting the slope (D_O/C_d) into Eq. (12) gives the $\beta_{3/1}$ values (Table 3). The $\beta_{2/1}$ and $\beta_{3/1}$ values less than 1 indicates that inhalation and dermal exposures result in lower doses to the liver than oral exposure does at the same potential dose. This is qualitatively in accordance with the previous findings (Weisel and Jo, 1996).

3.1.2. Estimations of $\alpha_{2/1}$ and $\alpha_{3/1}$

We calculated the AUC for the three exposure times (0.5, 5, and 50 min, see Table 3S of Supplementary Information) and the two concentration/dose levels (e.g., 0.014 and 0.14 mg/m^3 for inhalation concentrations) by using the PBPK model. The steady state concentrations were calculated for the continuous exposure of the same concentrations. The values of α_i were >0.99 for all exposure times, including short-term exposure of 0.5 min (Table 3S, Supplementary Information). Therefore, $\alpha_{2/1} = \alpha_{3/1} = 1$ (Table 3).

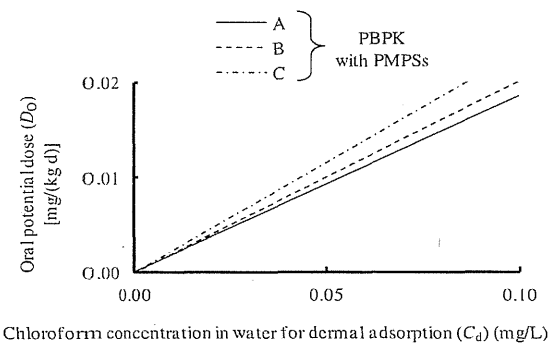

Fig. 3. Relationship between the oral potential dose of chloroform (D_O) and the chloroform concentration in water for dermal absorption (C_d) that yields equal chloroform metabolism rates. Lines were obtained by the PBPK model simulations with PMPSs A, B, and C.

3.2. Monte-Carlo and multi-exposure assessment

3.2.1. Distribution of total oral-equivalent potential dose

The solid line in Fig. 4 is the total oral-equivalent potential dose probability distribution function for chloroform when the chloroform concentration in drinking water equals 0.06 mg/L, which is the current Japanese DWQS. Vertical line segments indicate the median and the 95th percentile of the total oral-equivalent potential dose, 4.4 and 7.1 μg/(kg d), respectively. Even with the chloroform concentration in drinking water equal to 0.06 mg/L, the 95th percentile of the total oral-equivalent potential dose was below the TDI of 12.9 μg/(kg d). If the distribution is regarded as a probability distribution function for the population, the probability that the total oral-equivalent potential dose exceeds the TDI was 0.06%. Fig. 4 was obtained with EPS A. The probabilities were 0.04% and 0.001% for EPSs B and C, respectively (data not shown). Overall, the probabilities were less than 0.1%. There was hence sufficient margin between the total oral-equivalent potential doses and the TDI, even when people drank and used water with a chloroform concentration equal to the DWQS of 0.06 mg/L every day.

3.2.2. Allocation factor to drinking water

The result of the previous section revealed that there was sufficient margin even when the water was contaminated with chloroform up to the DWQS value. If the upper 95% confidence limit is assumed to be a useful indicator of high-end exposure (Nakanishi et al., 2006; Nitta et al., 2003; USEPA, 2000), a chloroform concentration that yields a 95th percentile estimate equal to the TDI may be protective for a majority of the population. Such a chloroform concentration was 0.110, 0.115, and 0.132 mg/L for EPSs A, B, and C, respectively (Table 4S of Supplementary Information). Accordingly, EPS A yielded the lowest chloroform concentration (the most conservative estimate). Fig. 5 shows the median and the 95th percentile estimates and their breakdown with respect to exposure routes, in% of the TDI. The chloroform concentration of 0.110 mg/L yields the median estimate of 8 μg/(kg d), while it yields the 95th percentile estimates of 12.9 μg/(kg d), that equals TDI. Calculated by Eq. (28), this ingestion results in a dose that corresponds to approximately 34% of the TDI for a 50-kg adult who drinks 2L of water per day. This figure of 34% is roughly 1.5 times greater than the default allocation factor of 20% that is currently applied in determining the Japanese DWQS.

$$G_v = \frac{\text{TDI} \times b_w \times A_f}{I_{water}} \tag{28}$$

For the median exposure (the 50th percentile estimate) component of the population, total indirect intake of chloroform via inhalation and dermal adsorption from water accounts for 28% of TDI (Fig. 5). This indirect intake was converted to an indirect water intake rate of about 1.65 Leq/d (liter-equivalent per day). Therefore, whereas the allocation factor associated with the direct water ingestion of 2 L/d is 34%, the allocation factor associated with the direct plus indirect water intake of 3.65 Leq/d [=(2 + 1.65) L/d] was 62% [=(34 + 28)%]. For a high exposure group, the direct plus indirect water intake rate was high and accordingly it occupied a high fraction of TDI. For the high-end exposure (the 95th percentile estimate) group, the direct plus indirect water intake rate was 5.86 Leq/d. Health Canada (2006) applies the allocation factor of 80% and the direct plus indirect water intake rate of 4.11 Leq/d. In our simulation the intake of 4.11 Leq/d occupied 70% of the TDI.

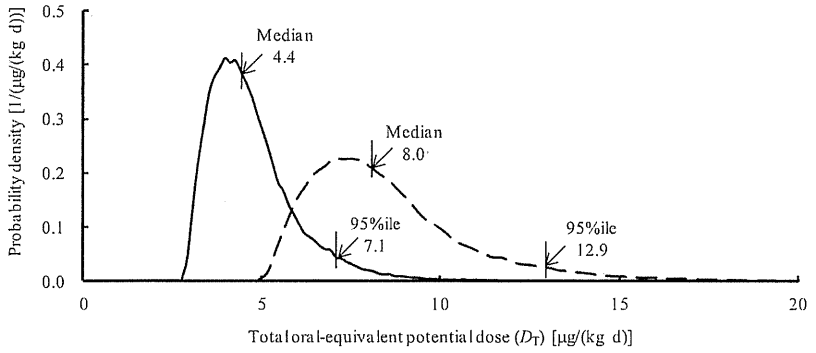

Fig. 4. The probability density function of the total oral-equivalent potential dose for chloroform based on the PBPK model with PMPS A, when chloroform concentration in drinking water was equal to the DWQS (0.06 mg/L; the solid line) and when the 95th percentile of the total oral-equivalent potential dose equals the TDI [12.9 μg/(kg d); the dashed line].

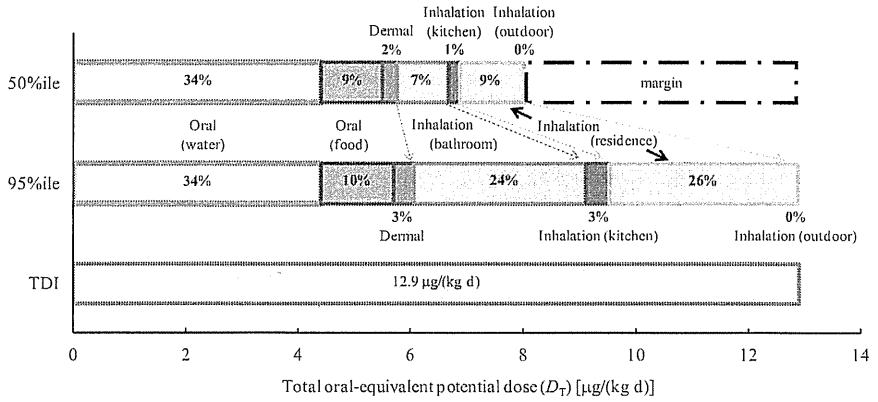

Fig. 5. Total oral-equivalent potential doses and their breakdowns. The upper and middle bars shows the median (50th percentile) and the 95th percentile estimates, respectively, when the chloroform concentration in drinking water was equal to the 0.110 mg/L. The 95th percentile estimate is equal to the TDI [12.9 μg/(kg d)], which is shown by the lower bar. The simulations were conducted with PMPS A.

These values (4.11 Leq/d and 70%) roughly coincide with those of Health Canada respectively although the TDI values used are different.

For the high-end exposure group, inhalation accounts for 53% of its total exposure, which came almost entirely from the chloroform that evaporated from water. Cooking food with water raised the exposure by 9% (from 1% to 10%, data not shown). Dermal absorption did not contribute much to the total exposure for the high-end exposure group; it was 3%. This very small contribution of dermal absorption is seemingly inconsistent with previous findings (Kim et al., 2004; Xu et al., 2002; Yanagibashi et al., 2010). The inconsistency could be due to three reasons. (1) It is due to the different inhalation scenarios: for the high-end exposure group chloroform concentrations in the bath room etc. were much higher than the concentrations for the median exposure group (the detail is explained later in this section). Actually, ratio of contributions from dermal/inhalation exposures was small: it was 1/8 for high-end exposure group whereas it was >1/4 for the median exposure group. (2) The inconsistency would be due to the difference in the skin permeability coefficient values used in the calculations. We used median values, whereas the previous study used high values (Table 2S). Our PBPK model parameters, including the skin permeability coefficient, were validated with data from human exposure (vide supra). (3) The inconsistency would also be due to the difference in relevant dose metrics because equivalent biologically-effective dose is not the same as equivalent exhaled concentration (Chiu and White, 2006). Conversely, PBPK modeling results were obtained in this regard on the basis of internal dose metrics (Haddad et al., 2006; Valcke and Krishnan, 2010). The data of the high contribution from dermal absorption reported elsewhere was not based on biologically effective dose but it was based on measurements of chloroform concentrations in exhaled breath a certain time after exposure (Jo et al., 1990). According to our PBPK simulations, the chloroform concentrations in exhaled breath after dermal exposure were higher than the chloroform concentrations in exhaled breath after inhalation exposure although two exposure levels were the same based on the AUC. Therefore, the dermal contribution to total exposure could be overestimated when the concentration in exhaled breath is used as a dose metric. The concentration in exhaled breath after exposure may therefore not be a dose metric appropriate for internal exposure estimation. Necessity of knowing the compound dose or its metabolic rate at the site of toxic action rather than measurable concentration in exhaled breath was reported previously (Weisel and Jo, 1996).

Fig. 5 also shows that between the median and the 95th percentile estimates, chloroform intakes via inhalation exposures (purple + blue + orange bars in the figure) were very different (The oral intake of chloroform from water was the same between the median and the 95th percentile estimates, because water consumption rates were assumed to be a constant (2 L/d) in the simulations). This difference in chloroform intakes via inhalation exposures made the 95th percentile estimate much larger than the median estimate. The large amount of exposure from inhalation could be caused by high chloroform concentrations in the air and/or long exposure times. In order to clarify which one (exposure concentration or exposure time) yielded the large amount of exposure, among the Monte Carlo simulation scenarios, we picked scenarios that produced the 95th percentile and the median

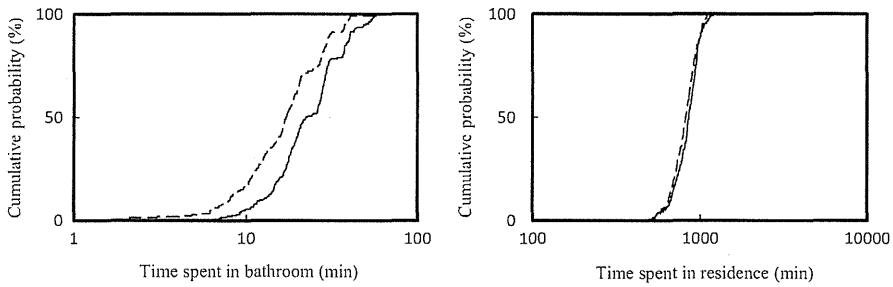

Fig. 6. Cumulative probability of the amount of time spent in the bathroom and in the residence for the two populations. The solid line represents the high-end exposure population (the 95th percentile population, total oral-equivalent potential dose = TDI). The dashed line represents the median exposure population.

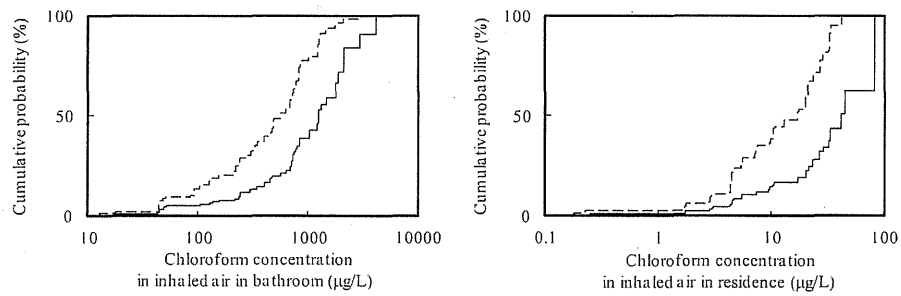

Fig. 7. Cumulative probability of chloroform concentrations in inhaled air in the bathroom and in the residence for the two populations. The solid line represents the high-end exposure population (the 95th percentile population, total oral-equivalent potential dose = TDI). The dashed line represents the median exposure population.

estimates. Exposure times were not so different between the two scenarios; one produced the 95th percentile, and the other produced median estimates, as shown in Fig. 6. However, chloroform concentrations were very different between the two scenarios, and chloroform concentrations in the scenario that produced the 95th percentile estimate were much higher than the concentrations associated with the median estimate, as shown in Fig. 7. Therefore, high chloroform concentrations in the air of the bathroom and residence would produce the high-end exposure group (the 95th percentile estimate). Such high chloroform concentrations could be caused by any activities that cause high chloroform evaporation from water and/or low ventilation in the rooms.

In this study, we considered variability of exposure scenarios in Monte Carlo inputs. Inter-individual variations are due to the variability of anthropometric properties and physiologically kinetic characteristics, varying as a function of age and gender, as well as the variability of exposure scenarios. Anthropometric and physiological model parameters were taken as variables for PBPK model simulation of several exposure scenarios (Beaudouin et al., 2010; Clewell et al., 2004; Haddad et al., 2006; Mörk and Johanson, 2010), and oral-equivalent liter of inhalation and dermal exposures and risk quotients for multi-route exposure were evaluated (Buteau and Valcke, 2010; Valcke and Krishnan, 2010). Distribution of anthropometric and physiological model parameters may be critical issue to better understand inter-individual variations on internal dosimetry. In our study, three model parameter sets were used after verification with experimentally observed data, but the distribution of the model parameters was not considered. Further research is needed for this issue. However, it should be noted that inter-individual variations resulting from variability of human toxicokinetics is an issue of chemical specific adjustment factor for deriving TDI in dose/concentration–response assessment (Valcke and Krishnan, 2011; WHO, 2005a). In our study, exposure assessment was conducted: route-to-route extrapolation was embedded to account the total exposure of chloroform from various routes and then inter-individual variations of exposure due to variability of exposure scenarios were calculated in order to compare exposures with TDI.

We conducted Monte Carlo simulations with considering variability of exposure scenarios, and source allocation to drinking water was calculated for high-end exposure populations. Therefore, this method is expected to reasonably result in a criterion that is protective of a majority of the population, as opposed to the simple subtraction methods in which high-end or average intakes be subtracted for every exposure source.

4. Conclusions

Multi-route exposures to chloroform via ingestion, inhalation, and dermal absorption were taken into account with the use of PBPK model simulations. By using the Monte Carlo simulation method, the probability distributions of total oral-equivalent potential doses resulting from variability of these exposures were estimated. With these distributions, maximum possible allocation factors for drinking water were then calculated and compared with the allocation factor currently applied in the DWQS.

The equation to estimate total oral-equivalent potential dose with a common endpoint of a single target organ [Eq. (9)] and its parameter values (Table 3) were derived to take into account multi-route exposures via ingestion (drinking water and food), inhalation, and dermal absorption. The probability distribution functions of total oral-equivalent potential doses indicated that even when the chloroform concentration in drinking water equals the DWQS value (0.06 mg/L), there was sufficient margin between the total oral-equivalent potential dose and the TDI. If the upper 95% confidence limit of the probability distribution was assumed to be a useful indicator of high-end exposure for protecting the majority of the population, a chloroform concentration that causes the 95th percentile estimate to equal the TDI is 0.11 mg/L. The oral intake of drinking water exhibiting a concentration of chloroform of 0.11 mg/L results in an oral dose that corresponds to 34% of the TDI

for a 50-kg adult who drinks 2 L/d of water. For the high-end exposure group the direct plus indirect water intake rate was 5.86 Leq/d, while it was 3.65 Leq/d for the median exposure group. High-end exposure was the result of high chloroform concentrations that were mainly caused by evaporation of chloroform from water in the residence including bathroom, rather than of long exposure times. Dermal exposure did not contribute much to the exposure for the high-end exposure group.

The exposure assessment method proposed in the present study could be a useful method for deriving allocation factors. In the future, assessments that take into consideration the distribution of daily drinking water intake (if available) should be conducted and compared with the present study.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2013.07.004.

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An antioxidant, N,N′-diphenyl-p-phenylenediamine (DPPD), affects labor and delivery in rats: A 28-day repeated dose test and reproduction/developmental toxicity test

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ABSTRACT

A 28-day repeated dose toxicity test and reproduction/developmental toxicity test for N,N′-diphenyl-p-phenylenediamine (DPPD) were conducted in [Crl:CD(SD)] SPF rats. Male and female rats were dosed with DPPD by gavage for 28 days at 0, 100, 300, or 1000 mg/kg bw/day or for a total of 42–46 days at 0, 8, 50, or 300 mg/kg bw/day. No significant adverse effects were observed in the repeated dose toxicity study up to 1000 mg/kg bw/day in both sexes. In the reproduction/developmental toxicity study, two females showed piloerection, hypothermia, and pale skin; one died and the other showed dystocia on day 23 of pregnancy at 300 mg/kg bw/day. Another female delivered only three live pups at 300 mg/kg bw/day. A significantly prolonged gestation period was observed at 50 and 300 mg/kg bw/day. The NOAELs of repeated dose toxicity and reproduction/developmental toxicity were considered to be 1000 and 8 mg/kg bw/day, respectively.

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1. Introduction

N,N′-diphenyl-p-phenylenediamine (DPPD; CAS: 74-31-7), a gray or dark gray powder, is used as a polymerization inhibitor and antioxidant (HSDB, 2012). The antioxidative activity of DPPD is implemented by the donation of a hydrogen to a radical derivative and breaking the autocatalytic cycle (Chemicalland21, 2012). DPPD is widely used in rubber, oils, and feedstuffs, especially for tires in the rubber industry due to its color and stability (Chemicalland21, 2012; HSDB, 2012). Occupational exposure to DPPD may occur through inhalation and dermal contact with this compound at workplaces where DPPD is produced or used (HSDB, 2012). DPPD was detected at a high rate in leachate samples from landfills containing plastic and rubber waste at concentrations of 0.1–13 ng/L (Hasegawa and Suzuki, 2005) and was found in air samples taken from one location at 0.002–0.009 ng/m^3 (MOE, 2005) in Japan. Therefore, exposure to DPPD via the environment is also anticipated.

The oral acute toxicity of DPPD is low with LD_{50} values of 2370 mg/kg bw in rats (Marhold, 1986) and 18,000 mg/kg bw in mice (Labor Hygiene and Occupational Diseases, 1966). A long-term feeding study also showed the relatively low toxicity of DPPD in rats (Hasegawa et al., 1989). Rats were fed a diet containing 0.5% or 2% of DPPD (194 or 857 mg/kg bw/day in males; 259 or 1024 mg/kg bw/day in females) for 104 weeks, and a dose dependent reduction in body weight gain (not associated with decreased food consumption) and a significant decrease in relative weight of the liver were observed in both sexes. Calcium deposition in the kidney in males was the only significant histopathological change. Erythrocyte count, hemoglobin, and hematocrit were significantly increased in the female treatment groups while they were dose dependently decreased in males. In this study, an autopsy was carried out 8 weeks after the cessation of DPPD administration; therefore, some difficulty exists in interpreting study results.

As for reproductive and developmental effects, a study in the 1950s showed that feeding doses of commercial grade of DPPD at 0.025%, 0.10%, 0.40%, and 1.60% prolonged the gestation period in

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; COX, cyclooxygenase; DPPD, N,N′-diphenyl-p-phenylenediamine; HPV, high production volume; NSAID, non-steroidal anti-inflammatory drug; OECD, Organisation for Economic Co-operation and Development.

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all treatment groups in rats (Oser and Oser, 1956). In this study, female rats were fed DPPD from 2 weeks before mating (a total administration period was not specified). Although fertility was not affected by the DPPD treatment, mortality of pups at birth was increased. In a later study by Marois (1998), daily doses of 20–40 mg of DPPD/animal from the 14th day of pregnancy prolonged the gestation period and caused stillbirths in rats (Marois, 1998). In these comparable studies, the fertility effects of DPPD in males were not assessed, and detailed study methods were not fully described.

DPPD is a high production volume (HPV) chemical with production or importation exceeding 1000 tonnes per year in Organisation for Economic Co-operation and Development (OECD) member countries and is listed in the most recent OECD HPV list for investigation of its environment and human health effects under the OECD Cooperative Chemical Assessment Programme (OECD, 2012). Although some early studies briefly showed DPPD toxicity, further reliable information was necessary to assess the human health effects of DPPD. Therefore, DPPD was selected as a target substance for the Safety Examination of Existing Chemicals in Japan. The present paper reports the results of the repeated dose toxicity screening test and reproductive/developmental toxicity screening test of DPPD in rats.

2. Materials and methods

The 28-day repeated dose study was performed at the Research Institute for Animal Science (RIAS) in Biochemistry & Toxicology (Kanagawa, Japan) in compliance with "the notice on the test method concerning new chemical substances (November 21, 2003, No. 1121002, Pharmaceutical and Food Safety Bureau, MHLW; No.2, Manufacturing Industries Bureau, METI; No. 031121002, Environmental Policy Bureau, MOE)" and "the standard for the test facility conducting tests concerning new chemical substances, etc. (November 21, 2003, No. 1121003 Pharmaceutical and Food Safety Bureau, MHLW; No. 3 Manufacturing Industries Bureau, METI; No. 031121004 Environmental Policy Bureau, MOE)". Animals were treated in accordance with "the regulations for animal experimentation in RIAS" and the test was conducted with the approval of "the Animal Care and Use Committee of RIAS".

The reproduction/developmental toxicity study was performed at the Food and Drug Safety Center, Hatano Research Institute (Kanagawa, Japan) in compliance with OECD Guideline 421 Reproduction/Developmental Toxicity Screening Test, along with the above described notice and standard. Animals were treated in accordance with "the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973)", "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No.88 of the Ministry of Environment, dated April 28, 2006)", "Guidelines for Proper Conduct of Animal Experiments (June 1, 2006)", and "the Guideline for Animal Experiment in Hatano Research Institute, Food and Drug Safety Center".

2.1. Animals

2.1.1. The 28-day repeated dose study

Male and female [Crl:CD(SD)] SPF rats were purchased from Atsugi Breeding Center, Charles River Japan, Inc., (Kanagawa, Japan). Five-week-old male and female rats (male: 152–172 g; female: 130–147 g) found to be in good health were selected for use. Male and female rats were distributed into four groups on a random basis. Animals were reared on a basal diet, Labo MR Stock; NOSAN corporation (Tokyo, Japan) and water *ad libitum* and were housed individually. Animals were maintained in an air-conditioned room at a room temperature of 21.9–23.0 °C, relative humidity of 55–61%, 12-h light/dark cycle, and 10 and more air changes per hour.

2.1.2. The reproduction/developmental study

Male and female [Crl:CD(SD)] SPF rats were purchased from Atsugi Breeding Center, Charles River Japan, Inc., (Kanagawa, Japan). Ten-week-old male and female rats (male: 370.2–446.9 g; female: 220.4–265.2 g) found to be in good health were selected for use. Vaginal smears of each female were examined, and only females showing a 4-day or 5-day estrous cycle were used. Male and female rats were distributed into four groups on a random basis. Animals were reared on a basal diet, CE-2; CLEA Japan, Inc. (Tokyo, Japan) and water *ad libitum* and were housed individually, except for mating and lactation periods. Animals were maintained in an air-conditioned room at a room temperature of 21.5–23.5 °C, relative humidity of 47–67%, 12-h light/dark cycle, and 15 air changes per hour.

2.2. Chemicals and dosing

2.2.1. The 28-day repeated dose study

DPPD (Lot No. 307605R, purity: 99.87%) was obtained from Seiko Chemical (Tokyo, Japan). Male and female rats (5 or 10 rats/sex/group) were dosed once daily by gastric intubation with DPPD at a dose of 0 (control: methylcellulose), 100, 300, or 1000 mg/kg bw for 28 days. After the dosing period, five rats per each sex at 0 and 1000 mg/kg bw/day were reared for 14 days without administration of DPPD as the recovery groups. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight.

2.2.2. The reproduction/developmental study

DPPD (Lot No. KWR0015, purity 100%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Male and female rats (13 rats/sex/group) were dosed once daily by gastric intubation with DPPD at a dose of 0 (control: sodium carboxymethyl cellulose), 8, 50, or 300 mg/kg bw. Males were dosed for a total of 42 days beginning 14 days before mating, and females were dosed for a total of 42–46 days beginning 14 days before mating to day 4 of lactation throughout mating and gestation periods. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight.

2.3. Observations

2.3.1. The 28-day repeated dose study

The first day of dosing was designated as day 1 of administration. All rats were observed daily for clinical signs of toxicity. Clinical signs in detailed observation in all animals were recorded one day before the administration period and once a week during the administration period. Sensory reactions for a sight reaction, hearing reaction, sense of touch reaction, pain reaction, pupil reflex, and righting reflex were recorded on day 27 of the administration period and on day 13 of the recovery period. Grip strength of fore and hind limbs was tested by a grip strength meter (MK-380R/FR, Muromachi Kikai Co., Ltd., Tokyo Japan) and spontaneous motor activity was recorded by an infrared-ray passive sensor system (SUPERMEX, Muromachi Kikai Co., Ltd., Tokyo Japan) on day 27 of the administration period and on day 13 of the recovery period. Body weight was recorded on days 1, 7, 14, 21, and 28 of the administration period, on days 7 and 14 of the recovery period, and on the day of necropsy. Food consumption was recorded once a week during both administration and recovery periods. Fresh urine was sampled from animals on day 22 of the administration period and on day 8 of the recovery period. Urine samples were tested for color, pH, protein, glucose, ketone bodies, bilirubin, occult blood, and urobilinogen.

Rats were euthanized by exsanguination under anesthesia 1 day after the final administration or 1 day after completion of the recovery period. External surfaces of the rats were examined. Abdomen and thoracic cavities were opened, and gross internal examination was performed. Blood samples were drawn from the abdominal aorta of fasted rats. Collected blood samples were examined for hematology by an automated hematology analyzer (XT-2000i, Sysmex Co., Kobe, Japan) and automatic coagulometer (KC-10A, Amelung, US). Serum biochemistry was tested by an automatic analyzer (JCA-BM8, JEOL, Tokyo, Japan) and automated electrolyte analyzer (NAKL-132, TOA electronics Ltd., Tokyo, Japan). The brain, thymus, heart, liver, spleen, kidney, adrenal gland, thyroid gland, pituitary gland, testis, epididymis, and ovary were isolated and weighed. Histopathological evaluations were performed on these organs in addition to the eye ball, spinal cord, lung, trachea, stomach, intestines, prostate, seminal vesicle, vagina, uterus, urinary bladder, sciatic nerve, lymph nodes, and bone marrow (femur) in control and highest dose groups.

2.3.2. The reproduction/developmental study

The first day of dosing was designated as day 1 of administration or day 1 of the premating period. The day of successful mating was designated as day 0 of the pregnancy period. The day on which parturition was completed by 11:00 was designated as day 0 of the lactation period. All rats were observed daily for clinical signs of toxicity. Body weight was recorded once a week during the administration period, and on the day of autopsy in males, and once a week during the pre-mating and mating periods, on days 0, 7, 14, and 21 of pregnancy, on days 0 and 4 of the lactation period and on a day of autopsy in females. Food consumption was recorded on days 1–2, 7–8, 13–14, 29–30, 35–36, and 41–42 of the administration period in males, and on days 1–2, 7–8, and 13–14 of the pre-mating period, on days 0–1, 7–8, 14–15, and 20–21 of the pregnancy period, and on days 3–4 of the lactation period in females. Daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the pre-mating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the 2-week mating period had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered as evidence of successful mating. Once insemination was confirmed, females were checked for signs of parturition before 11:00 from day 21 of pregnancy. Females were allowed to deliver spontaneously and nurse their pups until day 5 of the lactation period. Litter size and

numbers of live and dead pups were recorded, and live pups were sexed and individually weighed on days 0 and 4 of the lactation period. Pups were inspected for external malformations on day 0 of the lactation period.

Rats were euthanized by exsanguination under anesthesia on the day after the final administration in males and on day 5 of the lactation period in females. External surfaces of rats were examined. Abdomen and thoracic cavities were opened, and gross internal examination was performed. The testis, epididymis, prostate, and seminal vesicle were isolated from all males, and the testis and epididymis were weighed and histopathologically examined. The ovary, uterus, vagina, and mammary gland were isolated, and the ovary was weighed and histopathologically examined. Organs were stored in 10% formalin with 0.1 M phosphate buffer. Organs that showed gross pathological changes were histopathologically examined. The numbers of corpora lutea and implantation sites were counted. On day 5 of the lactation period, pups were euthanized by exsanguination under anesthesia, and gross external and internal examinations were performed.

2.4. Data analysis

To assess the homogeneity of data, parametric data were analyzed with Bartlett's test or the *F*-test. When homogeneity was recognized, data were analyzed using a one-way analysis of variance or the Student's *t*-test. Non-homogeneous data were analyzed with Kruskal–Wallis's rank test or the Aspin–Welch *t*-test. Non-parametric data were analyzed with Kruskal–Wallis's rank test or Mann–Whitney's U test. The Dunnett test or Dunnett type test was used to assess multiple comparisons. Fisher's exact test was used to assess categorical data. Five per cent levels of probability were used as the criterion for significance. Statistical analysis of pups was carried out using the litter as the experimental unit in the reproductive/developmental study.

2.5. Evaluation of bilirubin measurements by the diazo method

In the repeated dose study, bilirubin levels significantly increased without being related to toxicological effects in males. Because both bilirubin and DPPD contain –NH substitutes, the interference of DPPD with bilirubin measurements was anticipated. The interference of DPPD with bilirubin measurements was tested as follows. Serum samples were taken from untreated male rats, and 0.2 mL of DPPD at 0.001, 0.01, 0.1, and 1 mg/mL (1:1 aceton and dimethyl sulfoxide) was added to 0.5 mL serum of rats. In addition, rat liver S9 was added to DPPD at 0.1 mg/mL to test the interference of DPPD metabolites. Bilirubin levels were measured by the diazo method, the same method as that of the repeated dose study.

3. Results

3.1. The 28-day repeated dose study

No deaths were observed in any groups. There were no effects on the clinical observation, detailed clinical observation, sensory function, motor activity, body weight, or hematological findings. Food consumption significantly decreased in the fourth week at 300 mg/kg bw/day and in the third and fourth weeks at 1000 mg/kg bw/day in males (Table 1). Table 2 presents the urinary examination in rats given DPPD at the end of the administration period. Protein levels significantly decreased in all treatment groups, but this was not dose dependent and was considered to be due to spontaneously occurring higher levels in control groups.

As shown in Table 3, no effects were found in the hematological examination in rats dosed with DPPD for 28 days. Table 4 presents serum biochemistry in rats given DPPD at the end of the administration period. Total bilirubin significantly increased in all treatment groups at the end of the treatment period in males, but it was not observed at the end of the recovery period. When DPPD was added to rat serum, bilirubin levels measured by the diazo method increased in a concentration-related manner with or without the rat S9 mix (Table 5). Therefore, increased bilirubin levels in this study were considered to be due to interference by DPPD. In females, γ-GTP significantly decreased (0.63 IU/L) at 1000 mg/kg bw/day at the end of the administration period, but it was within the background data of the facility (0.31–2.06 IU/L) and was not considered to be toxicologically significant. This change was not observed at the end of the recovery period.

Table 6 shows the incidence of histopathological findings in rats. At necropsy, slight hydrometra in the uterus was found in one female at 300 mg/kg bw/day, and dilatation of the lumen was histopathologically observed in the uterus of this female at the end of administration period; however, no gross or histopathological effects in the uterus were observed at 1000 mg/kg bw/day. Relative and absolute weights of the thyroid gland in males and absolute weight of the kidney in females significantly increased at 100 mg/kg bw/day, but histopathological changes were not significantly different in these organs at the end of the administration period. No other effects were observed in organ weights in both sexes. In the histopathological examination, no significant changes were observed in both sexes.

3.2. The reproduction/developmental study

There were no effects on body weight, body weight gain, and food consumption. Neither death nor clinical toxicity was observed in males. One female in the 50 mg/kg bw/day group was sacrificed on day 9 of the administration period for incorrect operation at the time of the dosage. At 300 mg/kg bw/day, two females showed piloerection, hypothermia, and pale skin on day 23 of pregnancy. One of these two females died and the other was sacrificed due to dystocia on day 23 of pregnancy. Another female showing piloerection and pale skin delivered only three live pups. Nesting and nursing were not observed in this female, and this female was sacrificed on day 1 of lactation due to total litter loss. In addition, one female showed piloerection on day 23 of gestation, and another female showed pale skin on day 22 of gestation at 300 mg/kg bw/day. However, no abnormalities were found in their delivery.

No effects were observed in the organ weights of male and female rats given DPPD. The following gross pathological findings were observed in two females who died or were sacrificed on

Table 1
Body weight and food consumption in rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)	Male				Female			
	0	100	300	1000	0	100	300	1000
Body weight (g)								
Day 1	162 ± 6	164 ± 4	163 ± 7	162 ± 6	139 ± 5	138 ± 6	139 ± 7	139 ± 6
Day 7	213 ± 12	211 ± 6	216 ± 10	211 ± 10	162 ± 8	162 ± 5	159 ± 5	161 ± 9
Day 14	272 ± 16	273 ± 8	277 ± 10	268 ± 18	182 ± 11	182 ± 13	180 ± 10	181 ± 10
Day 21	325 ± 21	329 ± 15	329 ± 15	314 ± 24	203 ± 11	213 ± 16	202 ± 13	203 ± 15
Day 28	362 ± 27	368 ± 26	363 ± 14	349 ± 28	223 ± 11	230 ± 23	220 ± 13	220 ± 19
Food consumption (g/rat/day)								
Week 1	30 ± 4	30 ± 2	31 ± 3	29 ± 2	23 ± 3	21 ± 4	23 ± 2	22 ± 3
Week 2	32 ± 3	34 ± 1	33 ± 1	31 ± 3	21 ± 3	24 ± 3	22 ± 4	22 ± 2
Week 3	34 ± 3	36 ± 2	34 ± 2	32 ± 2*	23 ± 2	24 ± 4	22 ± 4	22 ± 3
Week 4	41 ± 4	38 ± 3	35 ± 3*	31 ± 3**	24 ± 3	24 ± 5	24 ± 1	23 ± 2

* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett test).

Table 2
Urinary findings of rats treated with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
No. of animals		5	5	5	5	5	5	5	5
Color	Colorless	1	0	0	0	0	0	0	0
	Pale yellow	4	5	5	5	5	5	5	5
Cloudy	Negligible	5	5	5	5	5	5	5	5
pH	7.0	4	1	2	3	0	0	0	0
	7.5	1	4	3	1	1	2	1	2
	8.0	1	0	0	0	4	1	3	3
	8.5	0	0	0	0	0	2	1	0
Protein[a]	±	0	5**	5**	4*	1	0	1	1
	1+	4	0**	0**	0*	4	4	3	3
	2+	1	0**	0**	1*	0	1	1	1
Occult blood	Negligible	5	5	5	5	5	5	5	5
Urobilinogen	0.1 (ehrlich unit/dL)	5	5	5	5	5	5	5	5
Bilirubin	Negligible	5	5	5	5	5	5	5	5

* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett test).
[a] Protein: ± (15–30 mg/dL), 1+ (30–100 mg/dL), 2+ (100–300 mg/dL).

Table 3
Hematological findings of rats treated with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
RBC	($10^4/\mu L$)	811 ± 37	773 ± 27	773 ± 41	800 ± 70	768 ± 28	739 ± 46	741 ± 57	777 ± 10
Hb	(g/dL)	15.6 ± 0.4	15.2 ± 0.4	15.1 ± 0.4	15.3 ± 1.0	14.9 ± 0.5	14.5 ± 0.5	14.2 ± 0.9	15.0 ± 0.3
Ht	(%)	47.4 ± 1.2	46.4 ± 1.0	46.3 ± 1.6	46.3 ± 2.9	44.4 ± 1.5	43.3 ± 1.4	42.5 ± 2.6	44.6 ± 0.4
MCV	(fL)	58.8 ± 2.4	60.0 ± 1.0	59.8 ± 2.9	58.2 ± 2.4	57.8 ± 1.9	58.6 ± 1.9	57.6 ± 1.9	57.6 ± 1.1
MCH	(pg)	19.3 ± 0.7	19.6 ± 0.3	19.6 ± 0.7	19.1 ± 0.7	19.4 ± 0.6	19.6 ± 0.7	19.2 ± 0.5	19.3 ± 0.3
MCHC	(%)	33.0 ± 0.2	32.7 ± 0.2	32.7 ± 0.5	33.0 ± 0.5	33.6 ± 0.3	33.4 ± 0.5	33.4 ± 0.4	33.6 ± 0.5
Ret.	(‰)	27.7 ± 5.4	28.5 ± 8.2	30.5 ± 6.0	32.4 ± 13.8	19.6 ± 1.8	23.6 ± 3.4	23.1 ± 4.9	19.5 ± 4.4
PT	(s)	13.1 ± 0.5	13.2 ± 0.2	13.1 ± 0.2	13.5 ± 0.4	13.3 ± 0.2	13.1 ± 0.2	13.0 ± 0.4	13.2 ± 0.4
APTT	(s)	20.9 ± 1.8	22.2 ± 1.1	20.5 ± 1.1	22.3 ± 0.6	18.4 ± 1.6	17.6 ± 1.1	17.7 ± 1.1	18.0 ± 0.8
Platelet	($10^4/\mu L$)	141 ± 10	130 ± 8	133 ± 16	150 ± 22	121 ± 16	122 ± 10	124 ± 10	132 ± 15
WBC	($10^2/\mu L$)	76 ± 25	76 ± 21	57 ± 12	69 ± 10	59 ± 23	39 ± 10	38 ± 9	44 ± 8

RBC: Red blood cell; Hb: Hemoglobin; Ht: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; Ret.: Reticulocyte; PT: prothrombin time; APTT: Activated partial thromboplastin time; WBC: White blood cells.

Table 4
Serum biochemistry in rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
No. of animals		5	5	5	5	5	5	5	5
LDH	(IU/L)	358 ± 153	289 ± 92	335 ± 123	349 ± 132	458 ± 119	341 ± 125	463 ± 233	406 ± 120
AST	(IU/L)	71 ± 8	84 ± 23	75 ± 4	72 ± 6	78 ± 8	67 ± 5	77 ± 13	94 ± 57
ALT	(IU/L)	34 ± 1	45 ± 20	38 ± 5	35 ± 2	28 ± 4	29 ± 4	30 ± 4	32 ± 13
ALP	(IU/L)	808 ± 78	819 ± 136	774 ± 52	818 ± 188	579 ± 48	426 ± 62	460 ± 152	452 ± 93
γ-GTP	(IU/L)	0.61 ± 0.27	0.40 ± 0.23	2.34 ± 4.08	0.42 ± 0.10	1.32 ± 0.48	0.81 ± 0.39	1.35 ± 0.08	0.63 ± 0.25*
T. protein	(g/dL)	5.82 ± 0.30	5.76 ± 0.29	5.82 ± 0.21	5.79 ± 0.11	5.96 ± 0.27	5.85 ± 0.10	5.88 ± 0.26	5.90 ± 0.18
Albumin	(g/dL)	2.90 ± 0.30	2.73 ± 0.23	2.86 ± 0.22	2.99 ± 0.17	3.12 ± 0.29	3.00 ± 0.09	3.06 ± 0.28	3.14 ± 0.18
Albumin/Globulin		0.99 ± 0.10	0.90 ± 0.06	0.97 ± 0.09	1.07 ± 0.09	1.11 ± 0.14	1.05 ± 0.06	1.09 ± 0.12	1.13 ± 0.07
T. cholesterol	(mg/dL)	62 ± 10	79 ± 19	81 ± 7	64 ± 13	79 ± 7	91 ± 15	75 ± 14	74 ± 10
Triglycerides	(mg/dL)	68 ± 26	64 ± 8	51 ± 17	56 ± 12	27 ± 11	27 ± 7	24 ± 10	18 ± 3
Glucose	(mg/dL)	152 ± 14	155 ± 11	145 ± 8	146 ± 7	128 ± 8	139 ± 7	133 ± 9	138 ± 22
BUN	(mg/dL)	14.4 ± 1.4	13.3 ± 1.8	12.3 ± 2.3	13.0 ± 1.2	15.5 ± 2.1	13.9 ± 2.2	14.0 ± 2.3	15.6 ± 3.2
Creatinine	(mg/dL)	0.38 ± 0.02	0.37 ± 0.04	0.40 ± 0.02	0.41 ± 0.03	0.46 ± 0.06	0.39 ± 0.05	0.39 ± 0.04	0.41 ± 0.06
T. bilirubin[a]	(mg/dL)	0.33 ± 0.05	0.53 ± 0.05**	0.60 ± 0.10**	0.61 ± 0.09**	0.26 ± 0.05	0.26 ± 0.01	0.29 ± 0.03	0.29 ± 0.04
Calcium	(mg/dL)	9.9 ± 0.5	9.8 ± 0.1	9.8 ± 0.2	9.8 ± 0.3	9.6 ± 0.4	9.5 ± 0.3	9.3 ± 0.2	9.5 ± 0.3
Phosphorus	(mg/dL)	8.2 ± 0.5	8.6 ± 0.4	8.4 ± 0.6	8.5 ± 0.6	7.0 ± 0.9	7.3 ± 0.5	6.8 ± 0.7	7.0 ± 0.5
Sodium	(mEq/L)	146 ± 1	147 ± 1	147 ± 0	146 ± 1	146 ± 1	147 ± 1	148 ± 2	147 ± 2
Potassium	(mEq/L)	4.93 ± 0.46	4.97 ± 0.16	5.15 ± 0.28	5.37 ± 0.41	5.04 ± 0.38	4.96 ± 0.52	4.94 ± 0.33	4.90 ± 0.32
Chloride	(mEq/L)	104 ± 2	105 ± 1	104 ± 2	105 ± 1	108 ± 1	108 ± 2	108 ± 3	108 ± 0

LDH: lactate dehydrogenase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen.
* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett type test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett type test).
[a] Interference of DPPD with bilirubin measurements in male rats is described in Sections 3 and 4 (see Table 5 also).

Table 5
Total bilirubin levels in male rat serum with or without the S9 mix measured by the diazo method.

DPPD (mg/mL)	Total bilirubin (mg/dL)	Total bilirubin (mg/dL)
	Without the S9 mix	With the S9 mix[a]
0 (serum)	0.28	0.34
0 (serum and vehicle[b])	0.33	0.35
0.001	0.32	
0.01	0.37	
0.1	1.03	1.04
1	1.17	

[a] Rat liver S9.
[b] Vehicle: acetone and dimethyl sulfoxide (1:1).

day 23 of pregnancy: hemorrhage in the lumen of the uterus, incomplete retention and red color in the lung, and dark red medulla and hardness on the kidney in both animals; hydrothorax in the thoracic cavity, attachment of red content in mucosa of the glandular stomach and recessed area, or red spots in the duodenum in either animal. In the histopathological examination, slight hemorrhage in the endometrium, and very slight edema, very slight foam cell accumulation in alveolus, and very slight capillary fibrinous thromboses in the lung were observed in the two females. The histopathological examination revealed no toxicological effects in other males and females.

Table 7 shows reproductive and developmental findings in rats given DPPD. One female at 8 mg/kg bw/day did not deliver pups by day 25 of gestation. An autopsy on day 26 of gestation revealed no implantations in this female. This female was excluded from the statistical evaluation of pregnant females. No changes attributable to the chemical were noted in the number of mated pairs, number of copulated pairs, copulation index, number of fertile males, fertility index, length of estrus cycle, pairing days until copulation, number of corpora lutea, number of implantations, implantation index, and number of pregnant females. Gestation lengths were significantly longer than the control group at 50 and 300 mg/kg bw/day.

Although no statistical significance was observed, the number of pups born, delivery index, number of live pups, birth index, and live birth index on day 0 of lactation dose dependently decreased. The number of live pups and viability index were also decreased on day 4 of lactation in treatment groups, especially at 300 mg/kg bw/day. No changes were observed in litter weights and body weights of pups on days 0 and 4 of the lactation period. No gross external or internal abnormalities were observed in pups.

4. Discussion

In the repeated dose study, no deaths were observed in any of the groups; there were no effects on the clinical observation, detailed

Table 6
Incidence of histopathological findings of rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)	Grade	Administration period				Recovery period	
		0	100	300	1000	0	1000
Male							
No. of animals		5	0	0	5	0	0
Lung							
Arterial mineralization	+	0	–	–	1	–	–
Foam cell accumulation	+	1	–	–	1	–	–
Heart							
Myocardial degeneration/fibrosis	+	0	–	–	1	–	–
Liver							
Microgramuloma	+	1	–	–	0	–	–
Extramedullary hematopoiesis	+	1	–	–	0	–	–
Kidney							
Hyaline droplet in the proximal tubular epithelium	+	4	–	–	5	–	–
Basophilic tubule	+	1	–	–	2	–	–
Thymus							
Hemorrhage	+	1	–	–	1	–	–
Spleen							
Extramedullary hematopoiesis	+	5	–	–	5	–	–
Deposition of a brown pigment	+	5	–	–	5	–	–
Prostate							
Interstitial lymphocytic infiltration	+	1	–	–	0	–	–
Female							
No. of animals		5	0	0	5	5	5
Lung							
Arterial mineralization	+	1	–	–	2	–	–
Osseous metaplasia	+	1	–	–	0	–	–
Liver							
Microgramuloma	+	2	–	–	1	–	–
Kidney							
Basophilic tubule	+	1	–	–	2	–	–
Solitary cyst	+	1	–	–	1	–	–
	++	1	–	–	0	–	–
Thymus							
Hemorrhage	+	0	–	–	1	–	1 (1)
Spleen							
Extramedullary hematopoiesis	+	5	–	–	5	–	–
Deposition of a brown pigment	+	5	–	–	5	–	–
Pituitary							
Remnant of Rahke's pouch	+	0	–	–	1	–	–
Utreus							
Dilatation of the lumen	+	0	–	1 (1)	0	–	–

Grade (+: slight change; ++: mild change; –: not applicable).
Parentheses indicate the number of rats examined.

Table 7
Reproductive and developmental findings in rats dosed with DPPD by gavage in the reproduction/developmental toxicity study.

Dose (mg/kg bw/day)	0	8	50	300
Number of mated pairs	13	13	12	13
Number of copulated pairs	13	13	12	13
Copulation index	100.0	100.0	100.0	100.0
Number of fertile males	13	12	12	13
Fertility index	100.0	92.3	100.0	100.0
Length of the estrous cycle in the pre-treatment period (days)	4.1 ± 0.3 (13)	4.2 ± 0.4 (13)	4.3 ± 0.5 (12)	4.1 ± 0.3 (13)
Length of the estrous cycle in the treatment period (days)	4.0 ± 0.0 (13)	4.1 ± 0.3 (13)	4.3 ± 0.5 (12)	4.2 ± 0.4 (12)
Pairing days until copulation	2.4 ± 1.3	2.7 ± 1.3	2.8 ± 1.5	2.7 ± 1.3
Number of corpora lutea	17.8 ± 2.2 (13)	18.4 ± 3.3 (12)	17.3 ± 1.3 (12)	16.9 ± 1.3 (11)
Number of implantations	15.9 ± 1.5 (13)	16.3 ± 2.7 (12)	16.2 ± 1.0 (12)	15.8 ± 1.9 (11)
Implantation index	90.7 ± 11.9 (13)	89.6 ± 16.3 (12)	94.0 ± 5.7 (12)	93.5 ± 8.1 (11)
Number of pregnant females	13	12	12	13
Number of pregnant females with live pups	13	12	12	11
Gestation length (days)	22.4 ± 0.5 (13)	22.8 ± 0.5 (12)	23.0 ± 0.0** (12)	23.0 ± 0.4** (11)
Day 0 of lactation				
Number of pups born	14.8 ± 2.1 (13)	14.8 ± 3.1 (12)	14.3 ± 1.5 (12)	13.7 ± 3.1 (11)
Delivery index	92.5 ± 7.5 (13)	90.7 ± 8.2 (12)	88.3 ± 8.7 (12)	86.7 ± 16.1 (11)
Number of live pups	14.7 ± 2.1 (13)	14.4 ± 2.7 (12)	13.8 ± 1.5 (12)	12.8 ± 4.1 (11)
Sex ratio	44.3 ± 18.3 (13)	39.4 ± 12.1 (12)	47.6 ± 14.1 (12)	48.1 ± 13.2 (11)
Birth index	92.1 ± 7.9 (13)	88.4 ± 7.1 (12)	85.8 ± 10.1 (12)	81.2 ± 24.7 (11)
Live birth index	99.5 ± 1.7 (13)	97.7 ± 5.4 (12)	97.2 ± 5.3 (12)	92.0 ± 20.7 (11)
Day 4 of lactation				
Number of live pups	14.5 ± 1.9 (13)	13.9 ± 2.6 (12)	13.8 ± 1.4 (12)	12.2 ± 5.0 (11)
Sex ratio	44.7 ± 18.2 (13)	39.4 ± 12.2 (12)	47.9 ± 14.3 (12)	48.0 ± 14.6 (10)
Viability index	99.1 ± 2.2 (13)	97.0 ± 8.5 (12)	99.5 ± 1.8 (12)	87.5 ± 30.0 (11)

Parentheses indicate the number of dams.
Copulation index = (number of copulated pairs/number of mated pairs) × 100%.
Fertility index = (number of fertile males/number of copulated pairs) × 100%.
Delivery index = (number of pups born/number of implantations) × 100%.
Birth index = (number of live pups on day 0/number of implantations) × 100%.
Live birth index = (number of live pups on day 0/number of pups born) × 100%.
Sex ratio = (number of male live pups/number of live pups) × 100%.
Viability index on day 4 of lactation = (number of live pups on day 4/number of live pups on day 0) × 100%.
* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett type test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett type test).

clinical observation, sensory function, motor activity, body weight, urinary examination, hematological findings, organ weights, or histopathological findings. In the blood chemistry examination, total bilirubin levels significantly increased in all treatment groups at the end of the treatment period in males; however, bilirubin and urobilinogen levels in urine did not increase. In addition, no related effects such as histopathological changes in the liver were observed. Because both bilirubin and DPPD contain –NH substitutes, the interference of DPPD with bilirubin measurements was anticipated. When DPPD was added to rat serum, bilirubin levels measured by the diazo method increased in a concentration-related manner with or without the rat S9 mix. Therefore, increased bilirubin levels in the present study were considered to be due to interference by DPPD. However, it is of interest that these effects were not observed in females.

Information on absorption, distribution, metabolism, and excretion (ADME) of DPPD is available in male rats (Umeniwa et al., 1985). DPPD dosed by an intraduodenal route was rapidly metabolized to DPPD glucuronide and was also suggested to be metabolized to hydroxylated DPPD. After a 6-day oral dosing, the total fecal excretion of DPPD was 55.4% (unchanged) and total urinary excretion of unchanged DPPD and glucuronide DPPD was 0.04%; unchanged DPPD was temporary detected in fat tissues. There is no information available on sex differences for the ADME of DPPD, and it is difficult to predict whether sex differences do indeed exist. Results of the present study may suggest that detectable DPPD or DPPD metabolites by the diazo method were very low in the serum of female rats for some reason.

In the reproduction/developmental toxicity study presented here, no effects were observed in male fertility function. The number of pups born, delivery index, number of live pups, birth index, and live birth index on day 0 of lactation dose dependently decreased, but they were not significant. No changes were observed in litter weights and body weights of pups on days 0 and 4 of the lactation period. We confirmed that gavage doses of DPPD significantly prolonged the gestation period in rats.

Marois (1998) investigated a possible mechanism of the prolonged gestation period caused by DPPD. When prostaglandin $F_{2\alpha}$, a regulator of uterus contraction, was injected into rats given 40 mg DPPD from the 14th day of pregnancy, observed adverse effects decreased. Prostaglandin E production was markedly inhibited by DPPD in rabbit kidney medulla slices (Fujimoto et al., 1984; Fujita et al., 1982). Prostaglanidins E_2 and $F_{2\alpha}$ induce uterus contraction (Parkington et al., 1999), and the prolonged gestation period was considered to be due to low prostaglandins levels caused by DPPD administration, similar to non-steroidal anti-inflammatory drugs (NSAIDs). If DPPD acts like NSAIDs, adverse effects such as gastrointestinal disturbances, antiplatelet activity, and kidney failure, known side effects of NSAIDs (Ejaz et al., 2004), can be caused by repeated doses of DPPD.

Prostaglandins are synthesized from arachidonic acid by cyclooxygenase (COX). Prostaglandins play an important role in modulating mucosal integrity and various functions of the gastrointestinal tract, and NSAIDs are known to damage the gastrointestinal tract by reducing these functions (Al-Saeed, 2012; Takeuchi et al., 2010). In the reproduction/developmental toxicity study, hemorrhage in the stomach and duodenum were observed in dead or sacrificed dams at 300 mg/kg bw/day, but no toxicologically significant effects were observed in food consumption. In the repeated dose study, food consumption significantly decreased at 300 and 1000 mg/kg bw/day in males; there is a possibility that DPPD affected the gastrointestinal tract in males. However, these changes

were not considered to be toxicologically significant because of high food consumption in the control group, no differences in body weights, and no gross- or histo-pathological effects in the gastrointestinal tract in the repeated dose study. This result was consistent with a 2-year feeding study in which no histopathological effects were found in the gastrointestinal tract (Hasegawa et al., 1989).

Prostaglandins also regulate platelet aggregation, and NSAIDs are known to inhibit platelet aggregation (Fabre et al., 2001). In the reproduction/developmental toxicity study, pale skin and hemorrhage in the uterus, stomach, and duodenum were observed in dead or sacrificed dams at 300 mg/kg bw/day. It is questionable if these observations may suggest inhibitory effects of platelet aggregation. In the repeated dose study, slight hydrometra in the uterus was observed in one female at 300 mg/kg bw/day at the end of administration period, but it was not dose dependent. In addition, heamorrhage in the thymus in one female was observed at 1000 mg/kg bw/day at the end of recovery period in the repeated dose study, but it was not observed at the end of administration period. Therefore, hydrometra in the uterus and heamorrhage in the thymus observed in the repeated dose study were considered to be incidental.

Gavage doses of DPPD showed weaker effects than a previously reported feeding dose study. In a feeding study by Oser and Oser (1956), the mean gestation period was significantly longer [22.9 days (22–24 days), 24.1 days (22–25 days), 25.2 days (23–29 days), and 24.7 (22–27 days) at 0.025, 0.10, 0.40, and 1.60% (7, 28, 113, and 450 mg/kg bw/day: conversion data from RTECS)] than that of the control group [22.1 days (21–23 days)] (Oser and Oser, 1956). An increased gestation length was associated with higher birth weights due to the longer growth period in the uterus and resulted in dystocia and stillbirths in the feeding study. It was considered that feeding doses of DPPD continuously inhibited prostaglandin synthesis, while gavage doses allowed prostaglandin synthesis intermittently.

In the 28-day repeated dose study, neither deaths nor dose-related adverse effects were observed up to 1000 mg/kg bw/day (the highest dose tested) in both sexes. Therefore, the NOAEL of repeated dose toxicity was considered to be 1000 mg/kg bw/day in rats. In the reproduction/developmental toxicity study, no adverse effects were found in male reproduction up to 300 mg/kg bw/day (the highest dose tested). However, significant longer gestation length was observed at 50 and 300 mg/kg bw/day in dams, and the NOAEL of reproduction/developmental toxicity was considered to be 8 mg/kg bw/day in rats.

Although a reproductive toxicity study is important for risk assessment, sometimes it is not conducted by predicting the effects from available repeated dose studies. When low reproductive toxicity is expected from repeated dose studies, only a prenatal developmental toxicity study can be conducted to observe the developmental effects of chemicals, but effects on fertility and parturition are not observed in this study. In case of DPPD, the results of a long term feeding study in rats (Hasegawa et al., 1989), and the current repeated dose study indicated very low toxicity; it could be expected that DPPD is unlikely to cause reproductive effects. However, our reproductive/developmental toxicity study showed a huge discrepancy in NOAEL with these repeated dose studies. Our experience suggests that conducting a reproduction/developmental study, which includes mating and parturition, is important for the risk assessment of reproductive toxicity.

In conclusion, the results of the current study sufficiently provide initial toxicity data for repeated dose and reproduction/developmental toxicities of DPPD. The NOAEL of repeated dose toxicity was considered to be 1000 mg/kg bw/day based on no adverse effects. The NOAEL of reproduction/developmental toxicity was considered to be 8 mg/kg bw/day based on a longer gestation length at 50 and 300 mg/kg bw/day.

Conflict of Interest

None of the authors have any conflicts of interest associated with this study.

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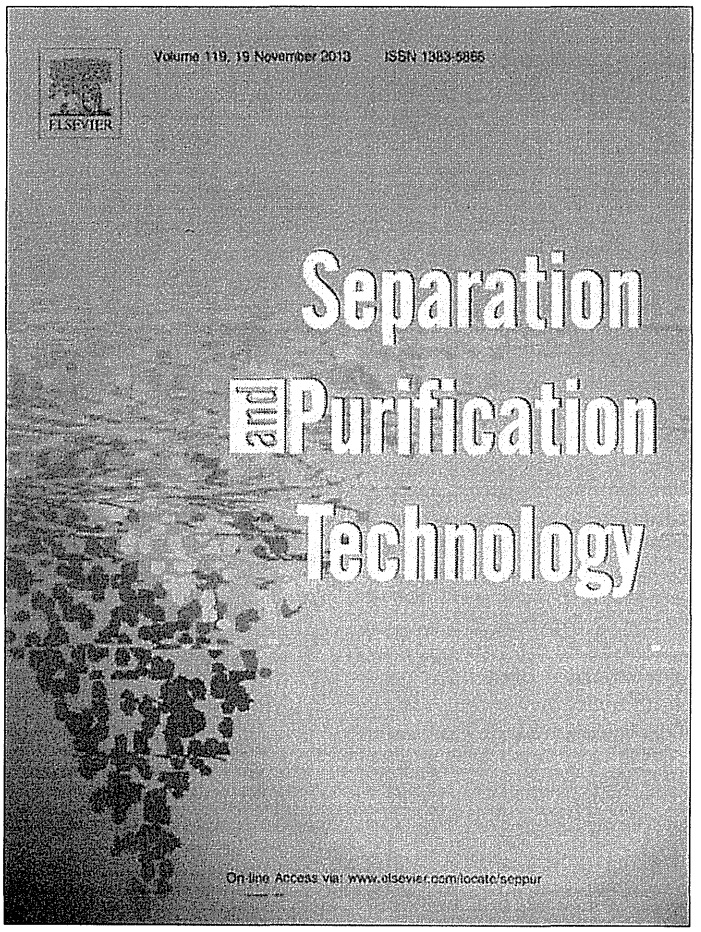

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