Measurement of Toluene Concentrations in the Blood of Fetuses of Pregnant Rats Exposed to Low Concentration Toluene Using Headspace-Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry

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In order to measure the level of toluene in the blood of fetuses of pregnant rats exposed to toluene, application of headspace-solid phase micro extraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) was studied. Pregnant rats from gestational day 15 (GD15) to GD19 were subjected to inhalational exposure to toluene for 90 min per day. They were obtained by Cesarean section on the fifth day of exposure (on GD19), and the level of toluene in the blood of mother rats and in fetuses were quantified using 5 μl blood. The levels of toluene in the blood of the fetuses in the groups exposed to toluene at 9 ppm and 90 ppm were 0.07 ± 0.03 μg/ml (n = 17) and 2.0 ± 0.51 μg/ml (n = 16) respectively, which were significantly higher than the blood level of the fetuses in the control group (0.02 ± 0.02 μg/ml, n = 12). Meanwhile, the blood concentrations of mother rats were 0.06 ± 0.06 μg/ml (n = 3, control group), 0.14 ± 0.03 μg/ml (n = 3, 9 ppm exposure group), and 3.5 ± 1.5 μg/ml (n = 4, 90 ppm exposure group), respectively, which were higher than those of the fetuses per unit blood volume.

Key words —— solid phase micro extraction, fetus, toluene, blood, headspace

INTRODUCTION

Since toluene is one of the most commonly used organic solvents, many studies have been conducted to examine its toxicity. One particular study clarified the lethal toxicity and acute effects on the central nervous system resulting from exposure to high concentration toluene.[1)] Regarding chronic toxicity, effects on the nervous system were also reported.[2, 3)] Toluene is also suspected to enhance chemical sensitivity (CS) or multiple chemical sensitivity (MCS).[4)] Having examined the effect of exposure to toluene on living organisms, we found additional toxicity of toluene including the following: Long-term exposure to low-concentration toluene caused upregulation of the information transmission circuits to occur within cells via enhanced development of N-methyl-D-aspartic acid (NMDA) receptor subunit 2B (NR2B) in the hippocampus of mice,[5)] and production of nerve growth factor modified interaction with the nervous system although significant disturbance in immunological memory function was not found.[6)] Since toluene is frequently detected in the atmosphere in general residential areas,[7, 8)] it is likely that people are exposed to it on a daily basis. Since toluene is used as a coating for construction materials and furniture, its indoor concentration tends to be high in newly constructed residences[9)] and extended or reconstructed buildings. The timing of such new construction work often overlaps with the birth of children, which is why it is necessary to examine the effect of exposure not only on adults but also on infants and fetuses, as well as the varying sensitivity of infants and fetuses to toluene. Although many experimental studies have been conducted using animals to examine the developmental toxicity of toluene,[10, 11)] all of those experiments used relatively high concentrations of toluene. In order to conduct studies on various end points, exposure experiments must be carried out at lower concentra-

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tions, and so the quantification method must be improved.

In order to examine the effect of exposure to toluene on fetuses, not only the concentration of toluene to which mother rats are exposed but also the level of toluene in the blood of individual rats must be measured. Sufficient amount of blood can be obtained from mother rats, but not be obtained from individual fetuses. Considering the volume to be used for assessing other effects, only several μl can be used for quantification. To measure the level of toluene in the blood, pretreatment of separating toluene from blood components is essential. However, if the sample volume is limited, the complex pretreatment operations decrease the recovery rate. On the other hand, the solid phase micro extraction (SPME) method, which has become widely used in recent years, allows specimens to be collected by adsorption from small amounts of solutions and pretreatment to be simplified. As part of the study to clarify the effect of toluene on fetuses, this study attempted to quantify toluene obtained from small amounts of blood samples from fetuses in the inhalational exposure experiment using pregnant rats, employing the SPME-GC-MS method.

MATERIALS AND METHODS

Reagent —— We used reagent grade toluene supplied by Wako Pure Chemical Industries (Osaka, Japan) in the inhalation exposure chamber. To quantify toluene for GC-MS, we used a standard solution from Supelco (Bellefonte, PA, U.S.A.), diluted as necessary with methanol (for testing residual pesticides; Wako Pure Chemical Industries).

Animals and Their Treatment —— The experiments using rats were conducted in compliance with the animal experiment ethics guideline of the National Institute of Environmental Studies, Tsukuba, Japan. Pregnant rats (Long Evans rats) were grown in an environment where they were able to take water and food *ad libitum*. The rats were subjected to inhalational exposure to toluene vapor continuously for 90 min per day for 5 days from gestational day 15 (GD15). The rats were divided into the control group, 9 ppm exposure group, and 90 ppm exposure group. Immediately after the completion of inhalational exposure on day 5 (GD19), they were anesthetized with Nembutal (at 90 mg/kg of weight), and then laparotomy was performed to take out fetuses. Blood of the mother rats was collected from the tip of their tail, while that of the fetuses was collected from incisions in the head using a 5 μl glass capillary tube (Drummond, PA, U.S.A.). To examine SPME conditions, sterilized blood of rats by Funakoshi Corporation (Tokyo, Japan) was used.

Inhalational Exposure of Pregnant Rats to Toluene —— Pregnant rats were subjected to inhalational exposure to toluene vapor at a concentration of 9 or 90 ppm for 90 min/day from GD15 to GD19 (day after mating = GD1). As controls, some pregnant rats were exposed to filtered clean air under the same conditions as for toluene exposure. Toluene vapor was generated by an organic solvent gas generator (Sibata Scientific Technology Ltd., Tokyo, Japan), diluted with filtered clean air to achieve the desired gas concentrations, and was then introduced into an acrylic exposure chamber. We adjusted the toluene concentration inside the exposure chamber according to the measurements taken using a portable GC-MS (Hapsite; Inficon, East Syracuse, NY, U.S.A.), quantifying the level using the leak-check mode with monitoring ion of $m/z = 92$. The carrier gas was nitrogen, and the probe temperature was 70°C. The mean levels of toluene gas concentration were 5.7 ± 4.9 ppb in the control group, and 9.10 ± 0.20 and 89.8 ± 1.00 ppm in the 9 and 90 ppm toluene groups respectively.

Conditioning SPME Fiber —— StableFlex fiber [10 mm length, 85 μm polydimethylsiloxane (PDMS)/divinylbenzene (DVB) coated, Supelco] was used as SPME fiber for the experiments. The SPME fibers were subjected to heat treatment before use at 250°C for 30 min while carrier gas was fed through the injection port of the GC. Pretreatment of SPME fibers was conducted immediately before each experiment, and the tip of the fibers was inserted into heat-treated septums, then the fibers were stored in screw-mouth test tubes containing activated carbon until the tests were conducted.

Quantification of Toluene in Blood —— With the direct dipping method employing the SPME, 400 μl of ultrapure water was poured into a vial equipped with a 2 ml septum, d_8-toluene solution (methanol solution, 10 μg/ml × 1 μl) as an internal reference solution and 5 μl of blood were added to the vial, and the SPME fibers were immersed in the solution. When the specified duration of immersion had elapsed, the SPME fibers were taken out, their surfaces were washed lightly with distilled water, the water was wiped off with Kimwipes, and then toluene was quantified with a GC-MS (QP5000,

Shimadzu Corporation, Kyoto, Japan). With the headspace method, 400 μl of ultrapure water and an agitator were placed in a crimp-top vial equipped with a 2 ml septum, collected blood was added to the vial, d_8-toluene solution (methanol solution, 10 μg/ml × 1 μl) was added, and the vial was capped and agitated. The vial was stored, cooled with ice, until it was used for the test. The SPME fibers were inserted into the headspace of the vial containing samples while the vial was agitated at room temperature, and when the specified time had elapsed, the fibers were exposed to the air within the vial to make them adsorb vaporized toluene. Quantitative analysis of the SPME fibers was performed immediately after the adsorption using a GC-MS (QP5000, Shimadzu Corporation) and a capillary column, DB-624 (30 m × 0.25 mmφ, 1.4 μm, J&W, CA, U.S.A.). The temperature was raised as follows: it was maintained at 50°C for one minute, increased to 160°C at the rate of 20°C/min., then up to 250°C at 30°C/min., and maintained at that level for one minute. Carrier gas, helium, was fed at the rate of 1 ml/min. The splitless method was used for feeding, and the temperature at the feeding port was kept at 250°C. Ionization was performed by the electron impact (EI) method, and the temperature of the ionization chamber was maintained at 230°C. We used the selective ion monitoring (SIM) for the measurement. The mass number of the target ion was $m/z = 91$, and the confirming ions were $m/z = 65$. The quantity of toluene detected by the SPME was calculated by conversion based on the analytical curve of the peak area obtained by the measurement employing the splitless feeding method. The limit of determination, 0.25 ng, was found from 10σ, which was obtained by repeated measurements conducted by the standard solution headspace SPME method. Blank samples, which contained no blood, were prepared for each experiment, and the quantified value was found by subtracting the blank value from the measured value.

RESULTS AND DISCUSSION

Review of SPME Method of Analyzing Toluene Contained in a Small Amount of Blood

Measurement methods employing SPME include the direct immersion method and the headspace method, so both methods were compared first. To sterilized blood of rats, d_8-toluene was added, and the recovery rates were compared. With the direct immersion method, the immersion time was set at 1, 2, and 5 min, and the recovery rate for each was as low as 8.9 ± 1.6%, 12 ± 4.3%, and 21 ± 8.6%, respectively. Since the SPME fibers directly contacted blood components with the direct immersion method, contamination was found on the surface. Specifically, although washing was performed using distilled water after the immersion in the blood, repeated measurements were likely to cause browning of the surface of fibers as well as reduction of adsorption efficiency. Based on these results of the preliminary studies, the headspace method was used to quantify toluene in the blood.

Condition Setting of the Headspace SPME Method

Schimming *et al.*[12] adopted the headspace SPME method to measure the toluene concentration in the blood of humans, and used 2 ml of blood and a 20 ml headspace vial. In this method, 5 μl of sterilized blood of rats was added to 2 ml of ultrapure water, and measurement was made within a 20 ml vial. When 1 ng d_8-toluene was added as reference, and the adsorption time was set to 1, 2, and 5 min, the recovery rates of d_8-toluene were unsatisfactory: 18 ± 4.2%, 16 ± 5.7%, and 45 ± 6.0%, respectively. To solve the problem, the vial was replaced with one equipped with a 2 ml septum and the volume of ultrapure water used for dilution of samples was altered to 400 μl. The recovery rates of d_8-toluene in this case were 60 ± 13%, 79 ± 11%, and 117 ± 7.9%, respectively (Fig. 1). If the amount of samples is limited as in the case of fetal blood samples measured this time, and the toluene concentration in samples is low, it is essential to improve the recovery rate, and a small-capacity vial was found to be effective for this purpose.

Next, 400 μl of distilled water was added to the 2 ml vial in the same manner, and the concentrations of toluene were measured in cases where no native toluene was added, and 1 ng and 5 ng of toluene were added. Table 1 lists the results obtained. With either concentration, approximately constant quantified values were obtained irrespective of the adsorption time. As shown in Fig. 1, the recovery rate was approximately 100% with 5 min of adsorption. However, with simplification of adsorption and the risk of contamination during operation taken into consideration, 2 min, which allows a sufficient recovery rate of over 60%, was adopted as the adsorption time.

Toluene exists in general households in con-

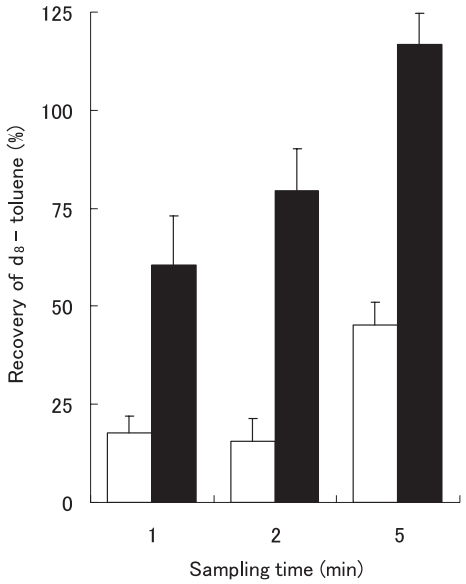

Fig. 1. Recovery Rate of d_8-Toluene by the Headspace-SPME Method (mean ± S.D.)

□: Five μl of blood was diluted with 2-ml distilled water, and measured with a 20 ml vial. ■: Five μl of blood was diluted with 400-μl distilled water, and measured with a 2 ml vial.

Table 1. Determination of Toluene by Headspace SPME Method

Spiked toluene (ng)	Sampling time (min)		
	1	2	3
0	0.42 ± 0.11	0.47 ± 0.02	0.30 ± 0.08
1	1.29 ± 0.11	1.31 ± 0.08	1.40 ± 0.08
5	5.38 ± 0.10	5.40 ± 0.14	5.41 ± 0.11

centrations of approximately several ppb, and it is therefore normal that it exists in general experimental environments in the same or higher concentrations. In fact, the daily toluene concentration in the laboratory where the head space SPME method performed was 3–5 ppb. As shown in Table 1, 0.3–0.5 ng of toluene was detected as the background value in the case in which no toluene was added. It was therefore necessary to perform the experiments as quickly as possible. Especially, when injecting the SPME needle into the head space vial or GC-MS port, SPME fiber contaminated by atmospheric toluene. To reduce the background toluene level, the needle should be injected into the vial as close as possible to the GC-MS port, and the laboratory should be ventilated with clean air by opening windows but that should be not affected the GC-MS measurement.

Figure 2 is a typical chromatogram of toluene in the blood of mother rat and fetus measured after the final exposure to toluene. When measurement was taken in the SIM mode, a good chromatogram was obtained without any disturbances. It was confirmed by matching with the retention time of the peak fixed to standard toluene's peak, and with the ratio of the quantitative and confirming ion (m/z = 65/91, standard products, 14–16%).

Results of Concentration Measurement of Toluene in Fetal Blood

Table 2 shows the weight of mother rats, fetuses, brains, and livers. There was no significant difference in the weight of mother rats, brains, and

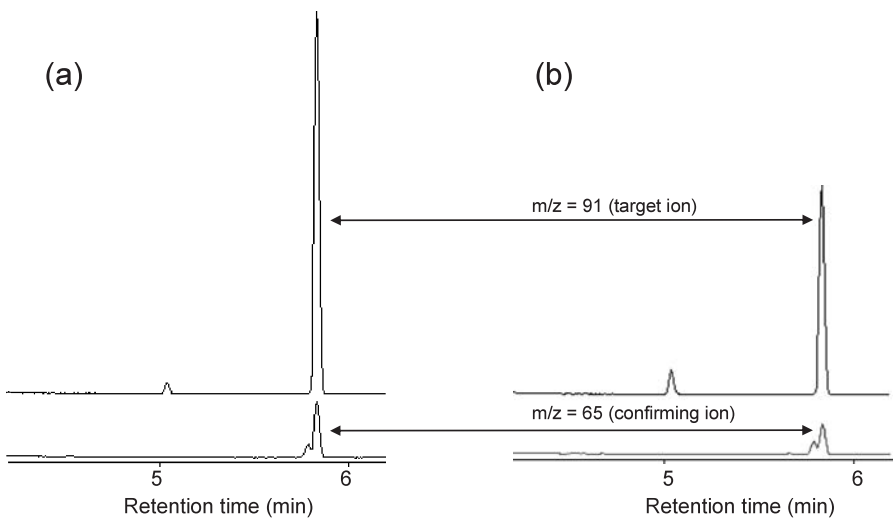

Fig. 2. Typical SIM Chromatograms of Toluene in the Blood of (a) Mother Rat and (b) Fetus

Table 2. Weight of Mother Rats and Fetuses, Weight of Brain and Liver

Exposure concentration (ppm)	Mother rat				Fetus			
	Weight (g)	Weight of brain (g)	Weight of liver (g)	*n*	Weight (g)	Weight of brain (mg)	Weight of liver (mg)	*n*
Control	324 ± 14	2.03 ± 0.1	13.5 ± 2.1	3	2.10 ± 0.10	168 ± 8.4	191 ± 39	12
9	306 ± 16	1.96 ± 0.1	11.8 ± 1.6	3	1.38 ± 0.11[a)]	128 ± 7.1[a)]	122 ± 22[a)]	17
90	295 ± 23	1.92 ± 0.1	11.7 ± 1.0	4	1.36 ± 0.10[a)]	128 ± 9.0[a)]	128 ± 20[a)]	16

a) Significant difference from the control group was found ($p < 0.05$).

Table 3. Toluene Concentration in the Blood of Mother Rats and Fetuses after Inhalational Exposure to Toluene

Exposure concentration (ppm)	Concentration in the blood (μg/ml)			
	Mother rat	*n*	Fetuses	*n*
Control	0.06 ± 0.06	3	0.02 ± 0.02	12
9	0.14 ± 0.03[a)]	3	0.07 ± 0.03[a)]	17
90	3.5 ± 1.5[a)]	4	2.0 ± 0.51[a)]	16

a) Significant difference from the control group was found ($p < 0.01$).

livers as well as the number of fetuses between the control group, which was exposed to clean air, and the groups exposed to toluene. On the other hand, significant differences were found in the weight of fetuses, brains and livers due to the exposure to toluene.

Table 3 lists the toluene concentrations in the blood of mother rats exposed to toluene of each concentration and of their fetuses. Blood was taken from four to six fetuses per mother rat subjected to inhalational exposure to toluene, and approximately the same number of fetuses was selected at random from the left and right uteri. It is known that the concentration of toluene in the blood of rats subjected to inhalational exposure to toluene quickly decreases.[13)] Therefore, blood was collected from mother rats at the midpoint of collection of blood from fetuses. However, the time required for collecting blood samples from one mother and six fetuses was less than 10 min, and no significant difference due to the difference of order of collection was found in toluene concentrations in the fetal blood.

As shown in Table 3, the higher the exposure concentration, the higher the concentration in the blood of the mother rats and fetuses. However, even if the exposure concentration was increased to 10 times as high as the original, the concentration in the blood was not 10 times as high, presumably because of the effect of the metabolism of mother rats in the case of low-concentration exposure. On the other hand, toluene was also detected in the blood of mother rats and fetuses of the control group at 0.06 ± 0.06 μg/ml and 0.02 ± 0.02 μg/ml respectively. The rats were grown not in a clean room where there was no trace of toluene, but in an environment where toluene was detected at the level of several ppb. The fact that toluene was detected in the blood of the control group reflects the effect of exposure to toluene in normal growing conditions. Meanwhile, the problem with this method was, as shown in Table 1, that the value of the blank sample was large, which was due to the existence of toluene in the experimental environment. Consequently, to decrease the limit of determination with this method, it is essential to reduce toluene concentrations, instead of improving measuring instruments, not only in the distilled water and solvents used for experiments but also in the environmental air in which animals are grown, and the air in the laboratory. Conversely, this method is considered to be very effective not only for toluene but also for other volatile organic compounds (VOCs) which are found in relatively low concentrations indoors.

Although a significant difference was not found due to the limited number of mother rats, the level of toluene in the blood tended to be higher in mother rats than in fetuses, which supports the findings of a past report on the exposure of pregnant rats to high concentration toluene.[14)] The concentration of toluene within organisms starts decreasing rapidly immediately after exposure, but it can accumulate easily in tissues high in lipid content.[14, 15)] The lipid content of fetuses is generally lower than that of adults,[16)] which may be one of the causes of the difference in the blood toluene level between mother rats and fetuses found by this study. Although blood lipid level was not measured in this study, it has been shown that lipid soluble compounds such as polychlorinated biphenyl (PCB) was parallel to the serum lipid level.[17, 18)] Therefore, it is better to mea-

sure blood lipid level in mother and fetus rat to determine the blood toluene concentration. Another factor involved may be the difference in toluene metabolizing activity between mother rats and fetuses, which is why future studies must take metabolic capacity into consideration.

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