

# Simultaneous Analysis of Urinary 2-Thiothiazolidine-4-carboxylic acid and Thiocarbamide as a Biological Exposure Index for Carbon Disulfide Exposure

Jaehoon Roh<sup>1</sup>, Chy Nyun Kim<sup>1</sup>, Nam Gu Lim<sup>1</sup>, Jung Hwan Chang<sup>1</sup>, and Yong Bong Cho<sup>2</sup>

## Abstract

The objectives of this study were to develop optimal analytic methods for detecting urinary 2-thiothiazolidine-4-carboxylic acid (TTCA) and thiocarbamide simultaneously and to evaluate the usefulness of these metabolites to a biological exposure index (BEI) for carbon disulfide (CS<sub>2</sub>) exposure. For this experiment, synthesized TTCA and thiocarbamide were used. The synthesized TTCA was identified by infrared spectrophotometer, nuclear magnetic resonance spectrometer and thin layer chromatography. The recovery rates of both metabolites were calculated to find the optimum analytical method. The amounts of urinary TTCA and thiocarbamide were measured by using an ultraviolet detector connected to high performance liquid chromatography (HPLC) after the administration of CS<sub>2</sub> (350, 700 mg/kg) into Sprague-Dawley rats intraperitoneally. The maximum absorbance wave lengths for TTCA and thiocarbamide were 272 and 236 nm, respectively. Ethyl acetate extraction with NaCl as a salting-out reagent was used as a simultaneous extraction method for these metabolites. HPLC conditions for these metabolites included using a NH<sub>2</sub> column, 50 mM KH<sub>2</sub>PO<sub>4</sub>: acetonitrile (85 : 15) and pH 3. Excreted amounts of urinary TTCA and thiocarbamide were increased significantly following CS<sub>2</sub> administration. TTCA, which was already adopted as a BEI for CS<sub>2</sub> by the American Conference of Governmental Industrial Hygienists (ACGIH), seems to be a more useful BEI for CS<sub>2</sub> exposure than thiocarbamide. However further studies are needed to increase analytical efficiency before thiocarbamide can be adopted as a BEI and to apply this analytic method for simultaneous analysis of these metabolites in workers exposed to CS<sub>2</sub>.

**Key Words:** Simultaneous analysis, carbon disulfide (CS<sub>2</sub>), 2-thiothiazolidine-4-carboxylic acid (TTCA), thiocarbamide, biological exposure index

## INTRODUCTION

A large amount of carbon disulfide (CS<sub>2</sub>) is used in the production process of rayon viscose fibers. Recently, there have been reports of workers with CS<sub>2</sub> poisoning which has presented a serious social problem in Korea.<sup>1</sup> In order to prevent the occurrence of CS<sub>2</sub> poisoning, the accurate measurement of its concentration in the worksite is needed. Environmental monitoring and biological monitoring methods have been used for evaluating the exposure of

workers dealing with certain environmental agents. In the case of environmental monitoring, this method only measures the level of agents in the work environment, leaving out the worker's physical workload, intensity of work, rate of inhalation, and the amount of skin absorption which could therefore alter the actual exposure of workers.<sup>2,3</sup>

Vasak et al.<sup>4</sup> used the iodine-azide test to analyze the amount of metabolite in the urine of workers exposed to CS<sub>2</sub>. However, this test is limited to places where the amount exceeds the air concentration of 50 mg/m<sup>3</sup>. The high background level in the urine of persons not exposed to CS<sub>2</sub> is also a limiting factor.<sup>4-7</sup> There have been numerous studies on the development and detection of metabolites in the urine of workers exposed to CS<sub>2</sub>. Pergal et al.<sup>8,9</sup> used infrared spectrometry (IR) and mass spectrometry to detect the metabolites 2-mercaptothiazolinone and thiocarbamide in the urine of workers exposed to CS<sub>2</sub> and

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<sup>1</sup>Institute for Occupational Health, Yonsei University College of Medicine, Seoul, <sup>2</sup>Department of Industrial Environment and Health, Yonsei University College of Health Science, Wonju, Korea

Address reprint request to Dr. J. H. Roh, Institute for Occupational Health, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea. Tel: 82-2-361-5354, Fax: 82-2-392-8622, E-mail: jhroh@yumc.yonsei.ac.kr

reported that a greater quantity of thiocarbamide was excreted. Van Doorn et al.<sup>10</sup> used <sup>1</sup>H-nuclear magnetic resonance spectroscopy (NMR) and gas chromatography/mass spectrometry to detect 2-thiothiazolidine-4-carboxylic acid (TTCA) and analyzed it quantitatively with high performance liquid chromatography (HPLC).<sup>10,11</sup> Furthermore, there has been research on expired air of rats administered with [<sup>14</sup>C] CS<sub>2</sub>.<sup>12</sup> In a more recent biological monitoring of CS<sub>2</sub>, measurement of TTCA in urine has been found to be more accurate than the iodine-azide test.<sup>13,14</sup> The American Conference of Governmental Industrial Hygienists (ACGIH) has recommended TTCA in urine as the biological exposure index (BEI) for CS<sub>2</sub> exposure.<sup>15</sup> However, despite the occurrence of CS<sub>2</sub> cases in Korea, there has been no systematic study on urinary metabolites as a BEI for CS<sub>2</sub> exposure.

This study focused on urinary TTCA and thiocarbamide as metabolites of CS<sub>2</sub> to determine the optimal experimental conditions for the simultaneous analysis of both metabolites and to evaluate the usefulness of these metabolite as a BEI for CS<sub>2</sub> exposure.

## MATERIALS AND METHODS

### Materials

Nine- to 10-week-old Sprague-Dawley rats weighing 195 to 235 g were used. The rats were purchased from the Division of Laboratory Medicine, Yonsei University College of Medicine (Seoul, Korea). Animal chow (Cheil Jedang, Seoul, Korea) and water were given *ad libitum* 1 week before the start of the experiment. An environment with a temperature of 24 ± 2°C and a humidity level of 65 ± 5 percent was maintained for the rats. The room's lighting system was a 12 hour light/dark cycle beginning at 9 : 30 a.m.

Acetonitrile (Fisher Scientific, Fairlawn, NJ, USA), KH<sub>2</sub>PO<sub>4</sub> (Junsei Chemical Co., Tokyo, Japan) and methanol (Fisher Scientific, Fairlawn, NJ, USA) used as chromatographic eluents were used after ultrasonification. The TTCA was available from Aldrich (Milwaukee, WI, USA) and Nihon Kankoshihiko (Okayama, Japan). We had already developed the synthetic method of TTCA<sup>16</sup> according to other reports.<sup>17,18</sup>

### Methods

The dosages of CS<sub>2</sub> (Merck, Darmstadt, Germany) administered to the Sprague-Dawley rats were one-fourth (350 mg/kg), and one-half (700 mg/kg) of LD<sub>50</sub> of CS<sub>2</sub>, 1,545 mg/kg body weight.<sup>19,20</sup> CS<sub>2</sub> was diluted with corn oil and injected 5 ml/kg body weight of rats once into the peritoneal cavity.<sup>21</sup> The control group was administered the same amount of corn oil. There were 5 rats in each experimental group. After administration of CS<sub>2</sub>, urine samples were collected after 12 hours and 24 hours in the metabolism cage and kept at -70°C before analysis.

The TTCA was synthesized by adding 10 g of L-cysteine (Fisher Scientific, Fairlawn, NJ, USA) and 8 g of NaOH (Kanto Chemical Co., Tokyo, Japan) to 60 ml of distilled water and mixed together. As well, 9.2 g of CS<sub>2</sub> was added and the mixture was stirred for 20 hours at 25°C. Then it was acidified up to pH 6 using HCl (Matsunoen Pharmaceutical Co., Osaka, Japan) and extracted by ethyl acetate (Tedia Company Inc., Fairfield, OH, USA). The extract was mixed with HCl and crystallized. It was re-crystallized with 6 N HCl and produced the TTCA in white crystals.<sup>17,18</sup> The yield amount of synthetic TTCA was 54%. The structure of synthesized TTCA was identified by IR (Model 435, Shimadzu, Japan) and <sup>1</sup>H-NMR (EM-360L, Varian, Australia) and thin layer chromatography (TLC) (Silica gel 60F-254, Merck, Darmstadt, Germany).

The recovery rate of TTCA and thiocarbamide in the rats' urine was calculated to find the optimum extraction method. Extraction method I consisted of introducing 5 ml urine sample into a test tube with 1.5 g of NaCl (Shinyo Pure Chemical Co., Osaka, Japan) as a salting-out reagent and 100 μl of 6 N HCl. After vortex-mixing for 30 seconds, 5 ml of ethyl acetate was added as an extraction solvent and shaken for 5 minutes. This was followed by centrifugation at 2,000 g for 5 minutes at 4°C. The upper ethyl acetate layer was transferred and capped in a test tube. The organic extraction was done twice with 5 ml of fresh ethyl acetate. The two organic extracts were then combined and mixed thoroughly. After centrifugation, the upper layer of extract was then transferred to a 50 ml beaker and dried at 40°C under a vacuum state. After drying, the residue was resuspended with 1 ml of 0.1% (v/v) H<sub>3</sub>PO<sub>4</sub> (Shinyo Pure Chemical Co., Osaka, Japan) and 20 μl was

injected for HPLC (Gilson, France) analysis. Extraction method II used Na<sub>2</sub>SO<sub>4</sub> (Shimaku's Pure Chemical, Osaka, Japan) as a salting-out reagent instead of using NaCl as in extraction method I. The rest of the procedure was the same as extraction method I. Extraction method III used NaCl as a salting out reagent and used diethyl ether (Showa Chemical Co., Tokyo, Japan) as an extraction solvent. Extraction method IV used Na<sub>2</sub>SO<sub>4</sub> as a salting-out reagent and used diethyl ether as an extraction solvent.

The ultraviolet (UV) absorbance spectrum of the standard reagents and the urinary metabolites were analyzed using the UV visible spectrophotometer (Model 160 A, Shimadzu, Japan). The amounts of urinary TTCA and thiocarbamide were measured by using a UV detector (Model 122, Gilson, France) connected to the HPLC. To find the proper HPLC columns for detection of two compounds, chromatographic separation by a C<sub>18</sub> column (Haisil HL C18 150×4.6 mm, Higgins Analytical Inc., Mountain View, CA, USA) and a NH<sub>2</sub>-column (Spherisorb S5 NH<sub>2</sub>, 4.6×250 mm, Waters, Tauton, MA, USA) was compared. The variable composition ratio of the mobile phase, acetonitrile and KH<sub>2</sub>PO<sub>4</sub>, were applied for better separation of these metabolites.

In order to compare the recovery rates and concentrations of urinary TTCA and thiocarbamide, the non-parametric Mann-Whitney U test was used.

## RESULTS

### Identification of synthesized TTCA

The synthesized TTCA was identified with IR spectrometry and <sup>1</sup>H-NMR. The infrared spectrum of the product showed the maxima for NH group vibration at 3,320 cm<sup>-1</sup>, the maxima for OH group vibration at 3,200 cm<sup>-1</sup>, and the maxima for carbonyl group vibration at 1,715 cm<sup>-1</sup> (Fig. 1). In the <sup>1</sup>H-NMR spectrum, the H-5<sub>A</sub> peak appeared at 3.86 ppm, the H-5<sub>B</sub> quartet peak at 3.59 ppm and the H-4 quartet peak at 4.84 ppm. The carboxyl group peak and the H-N singlet peak appeared at 10.40 ppm and 13.35 ppm, respectively (Fig. 2).

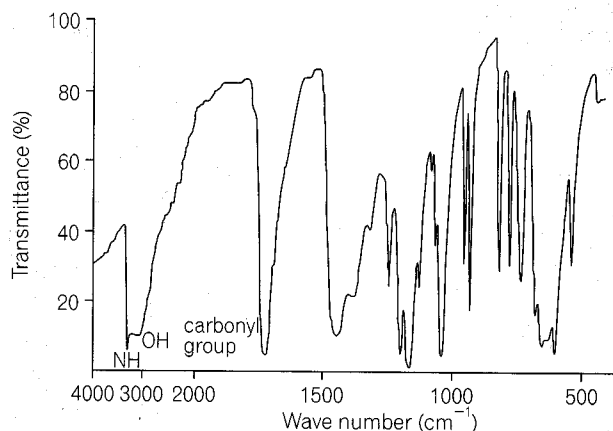


Fig. 1. Spectrum of 2-thiobiazolidine-4-carboxylic acid (TTCA) by infrared spectrometer.

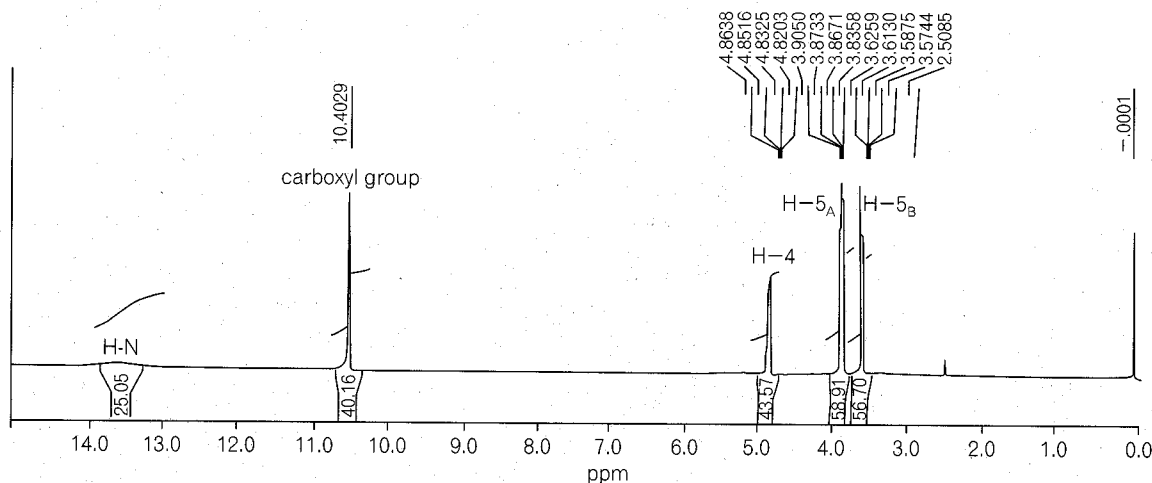


Fig. 2. Spectrum of 2-thiobiazolidine-4-carboxylic acid (TTCA) by <sup>1</sup>H-nuclear magnetic resonance spectroscopy (NMR).

### Optimum analytic conditions for TTCA and thiocarbamide

The UV spectrum of TTCA and thiocarbamide were measured by UV spectrometer. The maximum absorbance wave length ( $\lambda_{max}$ ) for TTCA was 272 nm and the maximum molar absorption coefficient ( $\epsilon_{max}$ ) was  $14,100 \pm 400 \text{ M}^{-1} \text{ cm}^{-1}$ . The maximum absorbance wave ( $\lambda_{max}$ ) for thiocarbamide was 236 nm, the maximum molar absorption coefficient ( $\epsilon_{max}$ ) was  $13,600 \pm 350 \text{ M}^{-1} \text{ cm}^{-1}$  (Fig. 3).

The recovery rate of TTCA and thiocarbamide by extraction method I were  $28.76 \pm 0.42\%$  and  $10.45 \pm 0.78\%$  respectively. The recovery rates of TTCA and thiocarbamide by extraction method II were  $29.10 \pm 6.49$  and  $5.28 \pm 0.53\%$ . The recovery rates by extraction method III were  $36.10 \pm 3.21$  and  $6.75 \pm 3.33\%$ . The recovery rates by extraction method IV were  $78.73 \pm 15.23$  and  $5.37 \pm 1.13\%$ . For TTCA,

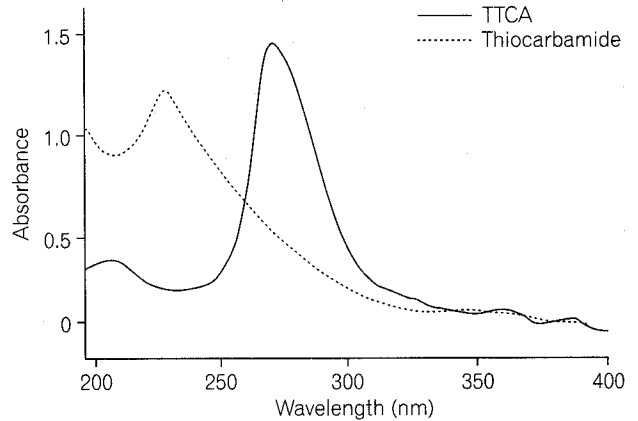


Fig. 3. Spectrum of 2-thiothiazolidine-4-carboxylic acid (TTCA) and thiocarbamide by ultraviolet visible spectrometer. TTCA;  $\lambda_{max} = 272 \text{ nm}$ ,  $\epsilon_{max} = 14,100 \pm 400 \text{ M}^{-1} \text{ cm}^{-1}$ . Thiocarbamide;  $\lambda_{max} = 236 \text{ nm}$ ,  $\epsilon_{max} = 13,600 \pm 350 \text{ M}^{-1} \text{ cm}^{-1}$ .

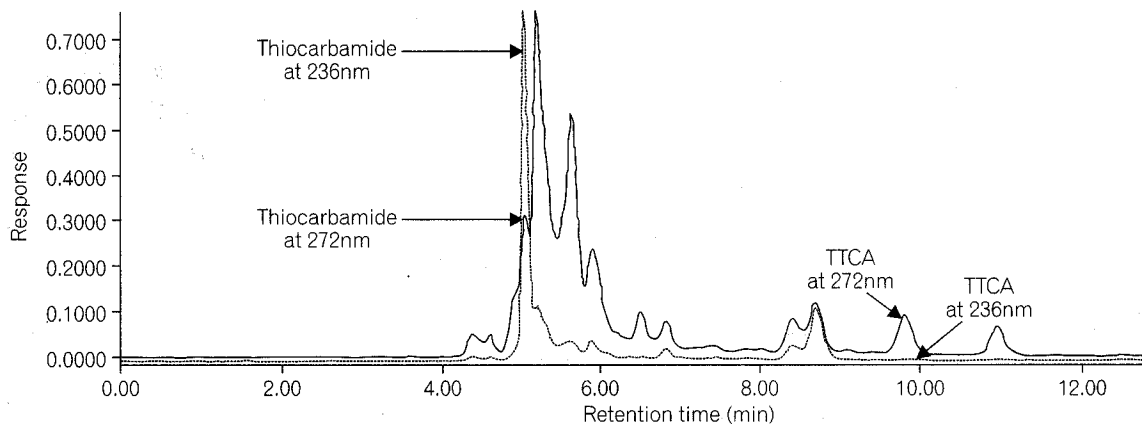


Fig. 4. High performance liquid chromatogram of 2-thiothiazolidine-4-carboxylic acid (TTCA) and thiocarbamide using a  $C_{18}$  column.

Table 1. Recovery Rate of 2-Thiothiazolidine-4-carboxylic acid and Thiocarbamide by Extraction Methods

Extraction methods	Solvents	Salts	Recovery rate (%)	
			TTCA	Thiocarbamide
I	Ethyl acetate	NaCl	$28.76 \pm 0.42$	$10.45 \pm 0.78^*$
II	Ethyl acetate	$\text{Na}_2\text{SO}_4$	$29.10 \pm 6.49$	$5.28 \pm 0.53$
III	Diethyl ether	NaCl	$36.10 \pm 3.21$	$6.75 \pm 3.33$
IV	Diethyl ether	$\text{Na}_2\text{SO}_4$	$78.73 \pm 15.23^*$	$5.37 \pm 1.13$

Values are mean  $\pm$  standard deviation.

\*  $p < 0.05$  from Mann-Whitney U test comparing other extraction methods.

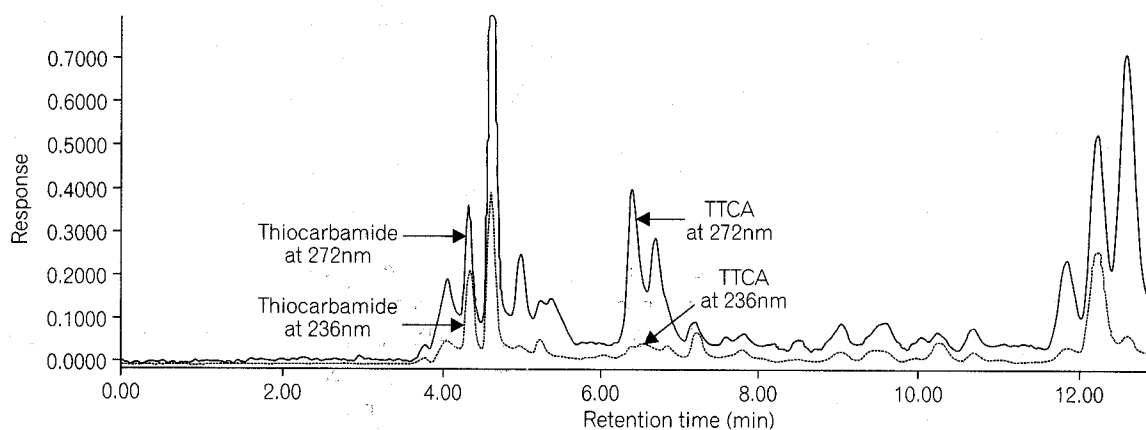


Fig. 5. High performance liquid chromatogram of 2-thiothiazolidine-4-carboxylic acid (TTCA) and thiocarbamide using a NH<sub>2</sub> column.

Table 2. Amount of Urinary 2-Thiothiazolidine-4-carboxylic acid and Thiocarbamide 12 and 24 Hours after Carbon Disulfide Administration

Metabolites	Sampling time (hours)	Doses (mg/kg body weight)	
		350	700
TTCA	12	483.4 ± 104.9	1539.1 ± 341.9*
	24	454.0 ± 169.7	1362.4 ± 330.1*
Thiocarbamide	12	55.0 ± 9.1	74.2 ± 5.4*
	24	47.6 ± 24.7	81.8 ± 5.9*

Values are Mean ± S.D. Unit is  $\mu\text{g/l}$ .

\*  $p < 0.05$  by Mann-Whitney U test comparing two doses of 350 and 700 mg/kg body weight.

the recovery rate by method IV (diethyl ether extraction with Na<sub>2</sub>SO<sub>4</sub>) was higher than the other methods. However, method IV had a low recovery rate of thiocarbamide and variable recovery rates of TTCA ( $\pm 15.23\%$ ). For thiocarbamide, the recovery rate by method I was higher than the other methods. Though the recovery rate of thiocarbamide was relatively low, extraction method I (ethyl acetate extraction with NaCl) was applicable for simultaneous extraction of TTCA and thiocarbamide (Table 1).

The C<sub>18</sub> and NH<sub>2</sub> columns were used to compare the separation of TTCA and thiocarbamide. The maximum wave length of thiocarbamide was 236 nm and the maximum wavelength of TTCA was 272 nm. The peaks of TTCA and thiocarbamide were seen on the chromatogram of urine samples. The TTCA was separated completely with the NH<sub>2</sub> column. However, separation of TTCA was not complete with the

C<sub>18</sub> column. For thiocarbamide, chromatographic separations were not complete with either column (Fig. 4 and 5). The HPLC conditions for simultaneous analysis of TTCA and thiocarbamide were by using the NH<sub>2</sub>-column (4.5 × 250 mm) and elution with 50 mM KH<sub>2</sub>PO<sub>4</sub> in acetonitrile (85 : 15) adjusted to pH 3.0 with HCl and a 1.0 ml/minute flow rate.

#### Urinary TTCA and thiocarbamide after the administration of CS<sub>2</sub>

The urine samples were collected at 12 hours and 24 hours after CS<sub>2</sub> was administered. The amounts of TTCA in the 700 mg CS<sub>2</sub>-administered group were 1539.1  $\mu\text{g/l}$  at 12 hours and 1362.4  $\mu\text{g/l}$  at 24 hours, while the 350 mg CS<sub>2</sub>-administered group were 483.4  $\mu\text{g/l}$  at 12 hours and 454.0  $\mu\text{g/l}$  at 24 hours after administration. The amounts of TTCA

were not different between 12 hours and 24 hours for both groups. However, the excreted amount of TTCA between administered doses were statistically different ( $p < 0.05$ ). The amounts of thiocarbamide were 55.0  $\mu\text{g/l}$  at 12 hours and 47.6  $\mu\text{g/l}$  at 24 hours in the 350 mg/kg administered group, and 74.2  $\mu\text{g/l}$  at 12 hours and 81.8  $\mu\text{g/l}$  at 24 hours in the 700 mg/kg administered group. The comparative amounts of excreted thiocarbamide were not statistically different between the hours of administration, but they were statistically different between the administered doses ( $p < 0.05$ ) (Table 2).

## DISCUSSION

An outbreak of CS<sub>2</sub> poisoning among workers at a rayon manufacturing plant in Korea has recently altered people to the problem and has increased concern about the accurate evaluation of work environments to prevent that kind of poisoning. Environmental monitoring of the workplace and biological monitoring of workers have been used in the evaluation of working environments: the former concerning only the working condition itself. Also, since the workload, the amount of inhalation, and the amount of skin absorption have not been taken into account, the results may be different from the actual amounts the workers were exposed to.<sup>3,22</sup> Thus, if possible, the evaluation of working environments must be conducted by simultaneously using both monitoring methods.

The excretion of CS<sub>2</sub> in urine may be biphasic. Recent pharmacokinetic studies have shown biphasic excretion with half-lives of 6 hours and 68 hours.<sup>23</sup> About 70 to 90% of the amount absorbed was metabolized and mainly excreted in the urine. The remaining 8-20% of the total absorbed dose may be eliminated unchanged in the exhaled breath and less than 1% through urination.<sup>24</sup> About 3% (range 2-6.5%) of CS<sub>2</sub> uptake was biotransformed to TTCA and excreted in the urine.<sup>23</sup> CS<sub>2</sub>-formed glutathione conjugate followed by an enzymatic degradation and ring-closure reaction were excreted in urine as TTCA.<sup>25</sup> 2-Mercaptothiazolinone was detected in the urine<sup>9</sup> and [<sup>14</sup>C] carbonyl sulfate (COS) and [<sup>14</sup>C] carbon dioxide was detected in the exhaled air of workers exposed to CS<sub>2</sub>.<sup>12</sup> CS<sub>2</sub> reacted with amino acid *in vivo* to form dithiocarbamate. Thus, CS<sub>2</sub> could

react with amine groups on critical cellular enzymes and thereby cause cellular damage. Dithiocarbamates are known to chelate metal ions such as copper and zinc. CS<sub>2</sub> exposure can decrease the activity of the copper-requiring enzyme dopamine- $\beta$ -hydroxylase.<sup>26</sup> This is related with the fact that two end-products of dopamine metabolism, homovanillic acid and vanillylmandelic acid, were decreased in chronic CS<sub>2</sub> poisoning cases.<sup>27</sup>

The UV spectrum showed that the maximum absorbance wavelength of TTCA was 272 nm. It was almost the same as the 273 nm reported by Van Doorn et al.<sup>28</sup> and the 276 nm by Rihimäki et al.<sup>23</sup> The analytic results using the NH<sub>2</sub> column (4.6  $\times$  250 mm) instead of the C<sub>18</sub> column used by Van Doorn et al.,<sup>10,28</sup> Rosier et al.<sup>11</sup> and Rihimäki et al.,<sup>23</sup> showed that there is no difference for the separation of thiocarbamide, but in the case of TTCA analysis, use of the NH<sub>2</sub> column proved more effective for separation. Most other investigators have found that one C<sub>18</sub> column was adequate for HPLC analysis.<sup>10,11,28-30</sup> Two reversed-phase HPLC columns, following diethyl ether extraction, were suggested to resolve the picomole level of TTCA from rat and human urine.<sup>31</sup> According to the comparison of the extraction methods for analyzing urinary metabolites, the ethyl acetate extraction method showed an acceptable recovery rate in both TTCA and thiocarbamide. The diethyl ether extraction method showed a very low recovery rate in thiocarbamide.

TTCA is produced after exposure to Captan and Disulfiram used in the management of alcoholism, and probably to other thiurams and dithiocarbamates often used as pesticides.<sup>25,32</sup> Humans treated with 250 mg of Disulfiram excrete up to 0.49 mg TTCA/g creatinine 10 hours after dosing.<sup>33</sup> TTCA was detected in the urine of individuals not exposed to CS<sub>2</sub>. Diet is considered as a non-negligible source of overestimation of CS<sub>2</sub> exposure. TTCA was detected in urine samples after ingestion of raw cabbage.<sup>34</sup> TTCA has also been identified in kidney bean seedlings treated with a dithiocarbamate fungicide, as well as in the dwarf pea and Sakurajima radish as an endogenous growth inhibitor.<sup>34,35</sup> CS<sub>2</sub> is highly soluble in fat and probably accumulates in the body. Obesity can cause a systematic change in TTCA excretion. This influence is noteworthy in considerably overweight employees with CS<sub>2</sub> exposure, and in such cases there is a risk of underestimating the expo-

sure.<sup>36,37</sup> TTCA excretion was not related to age, alcohol or nicotine consumption.<sup>38</sup> However, TTCA determination in urine currently constitutes the method of choice for assessing exposure to CS<sub>2</sub> and the use of these BEIs should be considered carefully.

CS<sub>2</sub> reacts with amino acid to form dithiocarbamates. These are partly metabolized to form thiocarbamide and 2-mercaptothiazolinone.<sup>28</sup> Pergal et al.<sup>9</sup> isolated thiocarbamide in the urine of workers exposed to CS<sub>2</sub> and identified it with thin chromatography and mass spectrometry. They reported in their study on CS<sub>2</sub> metabolism that thiocarbamide is probably the main metabolite present in urine of CS<sub>2</sub> exposed workers.<sup>9</sup> The enhancement of urinary excretion of thio compounds can be demonstrated in viscose workers, but this test lacks specificity.<sup>13,14</sup> Van Doorn et al.<sup>28</sup> reported that TTCA was the main metabolite in the urine of CS<sub>2</sub>-exposed workers found in the results of TLC data, UV spectrometry and HPLC. Still, the relationship between urinary TTCA and thiocarbamide is unclear.

The results of this analysis by HPLC indicated that TTCA excreted a greater amount in the urine than thiocarbamide. Also, the amount of urinary TTCA increased significantly according to the administered doses of CS<sub>2</sub>. After the administration of CS<sub>2</sub>, the results showed no difference in the excretion of TTCA and thiocarbamide depending on the urine collection time. TTCA has already been adopted as a BEI by the ACGIH. However, extraction of thiocarbamide still has analytic difficulties, but it does have possible uses as a BEI for CS<sub>2</sub> exposure. Based on the results of this study, it is necessary to develop a sensitive analytic method for urinary thiocarbamide determination. In addition, further research on the relationship between urinary TTCA and other metabolites of workers exposed to CS<sub>2</sub> should be pursued.

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