

Evaluation of urinary cyclohexanediols and cyclohexanol as biomarkers of occupational exposure to cyclohexane

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Abstract: Evaluation of urinary cyclohexanediols and cyclohexanol as biomarkers of occupational exposure to cyclohexane: Akito TAKEUCHI, *et al.* Osaka Occupational Health Service Center, Japan Industrial Safety and Health Association—Objec-

tives: The aim of the present study was to comparatively evaluate the usefulness of urinary cyclohexanediols (CHdiols-U) and cyclohexanol (CHol-U) as biomarkers of occupational exposure to cyclohexane (CH). **Methods:** Sixteen subjects (14 men and 2 women) were exposed to CH during proof-printing work. Personal exposure monitoring was conducted during the whole shift on the last working day of the week. The time-weighted average level of exposure to CH (CH-A) was measured using a diffusive sampler. Two urine samples were collected from each worker at different times during the same week: a baseline urine sample (before the first shift of the working week, after a 5-day holiday with no CH exposure) and an end-of-shift urine sample (after the last shift of the same working week, the same day personal exposure monitoring was conducted). CH-A, CHdiols-U and CHol-U were determined using a gas chromatograph-flame ionization detector. **Results:** The CH-A concentrations ranged from 4.5 to 60.3 ppm, with a geometric mean (GM) of 18.1 ppm. The GMs and ranges (in parenthesis) of the creatinine (cr)-corrected end-of-shift 1,2-CHdiol-U, 1,4-CHdiol-U and CHol-U concentrations were 12.1 (4.1–36.6), 7.5 (2.4–20.1) and 0.4 (0.2–1.0) mg/g cr, respectively. Both CHdiols-U at the end of the shift were significantly correlated with CH-A (correlation coeffi-

cients for 1,2-CHdiol-U and 1,4-CHdiol-U of 0.852 and 0.847, respectively). No correlation was observed between CH-A and CHol-U. **Conclusions:** CHdiols-U at the end of the last shift of the working week are suitable biomarkers of occupational exposure to CH, but CHol-U is not suitable.

(J Occup Health 2015; 57: 365–370)

Key words: Biomarker, Cyclohexane, Cyclohexanediol, Cyclohexanol, Occupational exposure

Cyclohexane (CH), a colorless liquid, is used to produce adipic acid and caprolactam. It is also used as a paint and varnish remover and as a solvent for cellulose ethers, waxes, resins, crude rubber and fats¹. CH has been considered a solvent of rather low toxicity. It causes depression of the central nervous system and has narcotic effects at high levels of exposure^{1,2}. Lammers *et al.*³ investigated the neurobehavioral effects of inhaled CH in rats and humans. In rats exposed for 8 hours/day for 3 consecutive days at 28,000 mg/m³ (8,000 ppm), there were slight reductions in psychomotor speed but minimal central nervous system effects. In human volunteers exposed for 4 hours to 86 or 860 mg/m³ (25 or 246 ppm) in 2 test sessions, there were no significant effects at the levels tested. Inhalation studies for 14 weeks of exposure in rats and mice resulted in a no-observed-effect level (NOEL) for subchronic toxicity in rats of 7,000 ppm based on the lack of adverse effects on body weight, clinical chemistry, tissue morphology and neurobehavioral parameters and a NOEL for subchronic toxicity in mice of 2,000 ppm based on hematological changes at 7,000 ppm⁴. The occupational exposure limits (OELs) (threshold limit value-time weighted average, TLV-TWA) for CH proposed

Received Dec 28, 2014; Accepted Apr 6, 2015

Published online in J-STAGE May 15, 2015

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by the Japan Society for Occupational Health (JSOH)⁵, the American Conference of Governmental Industrial Hygienists (ACGIH)¹¹ and the Deutsche Forschungsgemeinschaft (DFG)⁶ are 150, 100 and 200 ppm, respectively.

CH is absorbed mainly by inhalation and is distributed mainly in adipose tissue. It is metabolized to cyclohexanol (CHol), 1,2-cyclohexanediol (1,2-CHdiol) and 1,4-cyclohexanediol (1,4-CHdiol) in the human body (Fig. 1). These metabolites are excreted in the urine as both glucuronides and free non-conjugates⁷. The excreted amounts in urine of total absorbed CH were 0.5% as CHol, 23.4% as 1,2-CHdiol and 11.3% as 1,4-CHdiol⁸. Small amounts of unmetabolized CH and cyclohexanone (CHone) are also found in the urine of humans exposed to CH^{9, 10}. The elimination half-lives of 1,2-CHdiol, 1,4-CHdiol and CHol are 17.0 ± 5.2 (hours), 16.1 ± 3.9 (hours) and 1.5 (hours), respectively¹¹. The DFG proposed a biological tolerance value (Biologische Arbeitsstoff-Toleranzwerte, BAT) of 150 mg/g creatinine (after hydrolysis) for 1,2-CHdiol in urine collected at the end of the shift after several previous shifts, because 1,2-CHdiol is not a normal constituent of human urine; its concentration is higher than other major metabolites and proportional to external exposure^{7, 12}. However, studies on biological monitoring of occupational exposure to CH are limited, and biological monitoring of occupational exposure to CH has not been fully discussed.

The aim of the present study was to comparatively evaluate the usefulness of urinary CHdiols (CHdiols-U) and CHol (CHol-U) as biomarkers of occupational exposure to CH. To examine the exposure-excretion relationship between personal exposure and these biomarkers, we conducted a survey for workers exposed to CH during proof-printing work.

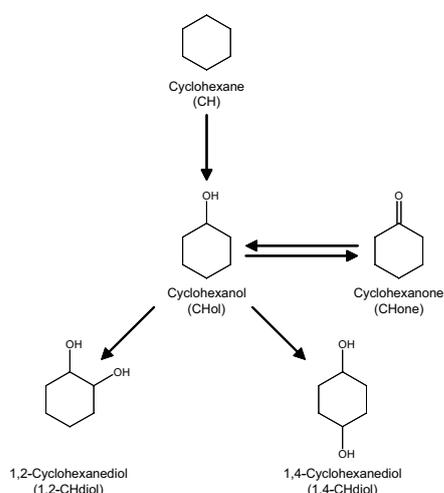


Fig. 1. Metabolism of cyclohexane in humans⁷.

Subjects and Methods

Ethical approval of the study protocol

This study was approved by the Ethics Committees of the Graduate School of Medicine, Osaka City University (approval number 2649). Informed consent was obtained from all subjects involved.

Subjects

The subjects of the study were 16 Japanese workers (14 men and 2 women) aged 18–61 years (mean \pm SD: 35.9 ± 13.2 years). They were engaged in proof-printing work for 8 hours a day and printed several proof sheets using red, black and yellow ink sequentially. When changing the ink color, they used an organic solvent containing CH to remove ink from the rubber transcription roller (called the “blanket”) and were exposed to CH. The proportions of CH, ethanol and 1-methoxy-2-propanol in this blanket cleaning solvent were 50–60%, 10–20% and 20–30%, respectively. The concentrations of these chemicals in the workplace air were measured by stationary sampling using a Tedlar bag for CH and 1-methoxy-2-propanol and a diffusive sampler for ethanol. The samples were analyzed by a gas chromatograph equipped with a flame ionization detector (GC-FID). The geometric mean (GM) concentrations of CH, ethanol and 1-methoxy-2-propanol in the workplace air were 13, 4 and 2 ppm, respectively. The workers used protective gloves when cleaning the blanket, so their skin exposure seemed to be negligible. They did not use protective masks.

Personal exposure monitoring and analysis of CH

Personal exposure monitoring was conducted during the entire work shift (8 hours) on the last working day of the week (on Friday). In order not to exceed the capacity of the sampler, the time-weighted average (TWA) level of exposure to CH (CH-A) was measured separately during the first and second half of the work shift with a diffusive sampler containing a charcoal adsorbent pad (3M 3500 organic vapor monitor, 3M, St. Paul, MN, USA) set up near the breathing zone of each worker.

Sampling and analysis were carried out according to the manufacturer’s guides for the 3M 3500 organic vapor monitor¹³. The CH collected by the diffusive samplers was desorbed into 1.5 ml of carbon disulfide containing *tert*-butylbenzene as an internal standard (500 ppm). A 1- μ l aliquot of the desorption solution was injected into the GC-FID. The GC-FID system used was a 7890A Gas Chromatograph (Agilent Technologies, Palo Alto, CA, USA). The column was a 60 m \times 0.25 mm ID DB-624 capillary column with a 1.4- μ m film thickness (Agilent Technologies, Palo

Alto, CA, USA). The oven temperature was set at 40°C for 1 minute and then increased to 230°C at a rate of 10°C/min. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The injection port and detector temperatures were maintained at 250°C. The sample was injected in pulsed split mode (pulse pressure, 45 psi; pulse time, 1 minute; split ratio, 20:1). The concentration of CH-A was calculated using the sampling rate supplied by the manufacturer. The 8-hour TWA was calculated as:

$$\text{8-hour TWA (ppm)} = [(C_f \times t_f) + (C_s \times t_s)] / 8$$

C_f and C_s are the TWAs of the first and second half of the work shift (ppm), respectively, while t_f and t_s are the sampling times of the first and second half of the work shift (hours), respectively. The limit of quantification (LOQ) of CH-A was 1.0 ppm.

Urine collection and analysis of urinary CHdiols and CHol

Two spot urine samples were collected from each worker during the same week: a baseline urine sample and an end-of-shift urine sample. The baseline urine sample was collected before the first shift of the working week after a 5-day holiday with no CH exposure (i.e., on Monday morning). The end-of-shift urine sample was collected at the end of the last shift of the same working week (i.e., on Friday evening, the same day personal exposure monitoring was conducted).

The analyses of CHdiols-U and CHol-U were carried out according to the method used by Mraz et al.¹⁴, with minor modifications. In brief, 5 ml of the urine sample was hydrolyzed with 1 ml of 50% H₂SO₄ containing 1% paraformaldehyde in a boiling water bath for 60 minutes. After the sample cooled to room temperature, 2 ml of NaOH solution (43 g/100 ml) and 5 g of K₂CO₃ were added, and the sample was

then shaken for 2 minutes. Extraction was performed with 1 ml of ethyl acetate containing *p*-dichlorobenzene (100 mg/l) as an internal standard, and then the sample was centrifuged at 3,000 rpm for 10 minutes. A 1- μ l aliquot of the extraction layer was injected into the GC-FID. The GC-FID conditions were the same as those for CH-A except for the column (DB-1: 60 m \times 0.25 mm ID, 1.0- μ m film thickness; Agilent Technologies), the oven temperature program (40°C for 1 minute, increased to 280°C at 10°C/min) and the split ratio (5:1). The method was validated according to US FDA guidance¹⁵. The accuracy and precision of the method met FDA criteria. Calibration curves showed linearity in the range of 1–100 mg/l for each analyte, with correlation coefficients of >0.997. For each analyte, the LOQ was 1.0 mg/l. The recoveries were 84–88%, 80–83% and 56–69% for CHol, 1,2-CHdiol and 1,4-CHdiol, respectively. Intraday accuracy and precision were 96.3–110.4% and 1.1–2.9%, respectively. Interday accuracy and precision were 95.6–108.8% and 1.3–5.2%, respectively.

Statistical analysis

The data below the LOQ for each urinary metabolite were set to half the LOQ (0.5 mg/l). The Student's *t* test for paired samples was used to evaluate differences between urinary levels of each metabolite in the baseline urine and end-of-shift urine sample. Simple regression analysis was employed to examine the exposure-excretion relationship.

Results

Personal exposure concentrations of CH

Personal exposure levels are expressed as the 8-hour TWA in Table 1. All subjects were exposed to CH concentrations lower than half the OEL proposed by the JSOH.

Table 1. Personal exposure concentrations of cyclohexane (CH-A) and urinary concentrations of cyclohexanediols (CHdiols-U) and cyclohexanol (CHol-U), n=16

	Correction for	Unit	Baseline				End of shift			
			GM	GSD	Range	n<LOQ	GM	GSD	Range	n<LOQ
CH-A	–	ppm			—		18.1	2.1	4.5 – 60.3	0
1,2-CHdiol-U	None	mg/l	1.0	2.3	<1.0 – 5.1	9	18.5	2.0	5.3 – 40.9	0
	Creatinine	mg/g cr	0.7	2.0	0.3 – 2.5	—	12.1	1.7	4.1 – 36.6	—
1,4-CHdiol-U	None	mg/l	0.7	1.7	<1.0 – 3.2	10	11.5	2.1	3.0 – 27.2	0
	Creatinine	mg/g cr	0.5	1.9	0.3 – 2.5	—	7.5	1.8	2.4 – 20.1	—
CHol-U	None	mg/l	0.5	1.0	<1.0 – <1.0	16	0.6	1.6	<1.0 – 2.0	12
	Creatinine	mg/g cr	0.3	2.4	0.1 – 2.5	—	0.4	1.8	0.2 – 1.0	—

GM, geometric mean; GSD, geometric standard deviation (dimensionless); LOQ, limit of quantification (1.0 ppm for CH-A and 1.0 mg/l for CHdiols-U and CHol-U, respectively). n<LOQ: Number of values below LOQ. The data below the LOQ for each urinary metabolite were set to half the LOQ (0.5 mg/l) in the calculations of GM and GSD.

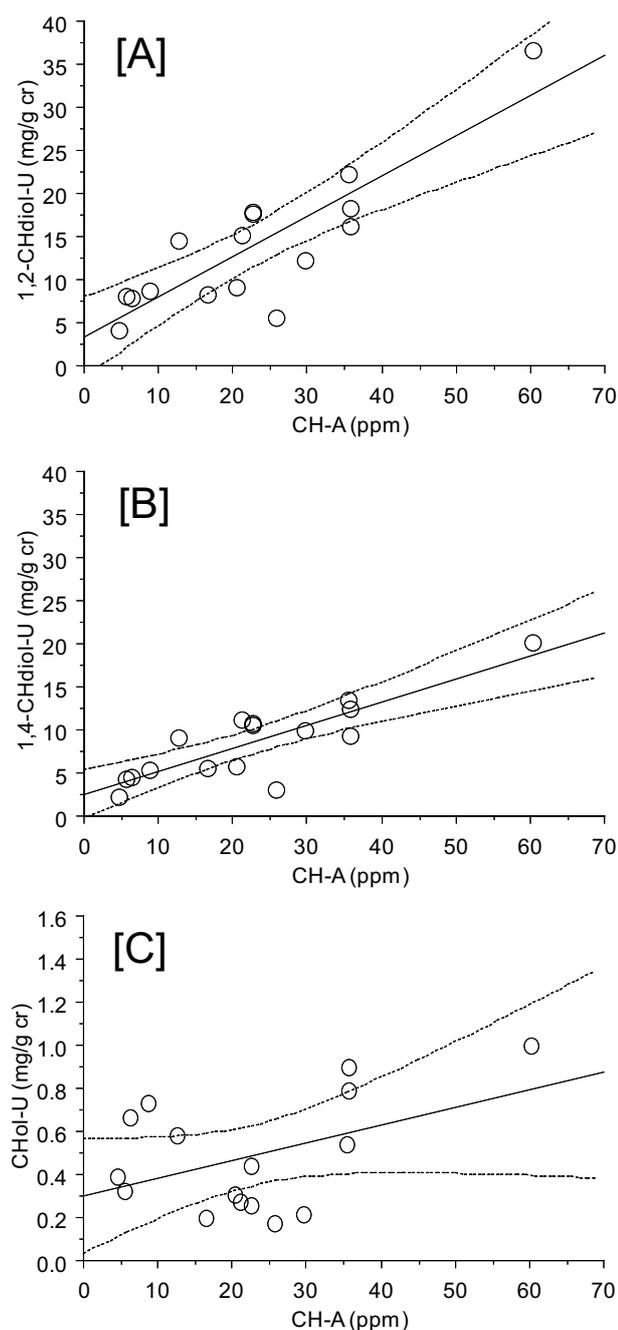


Fig. 2. Correlation between personal exposure concentration of cyclohexane (CH-A) and urinary concentrations. [A] 1,2-cyclohexanediol (1,2-CHdiol-U), $y=0.466x + 3.354$, $r=0.852$, $p<0.0001$; [B] 1,4-cyclohexanediol (1,4-CHdiol-U), $y=0.268x + 2.593$, $r=0.847$, $p<0.0001$; and [C] cyclohexanol (CHol-U), $y=0.008x + 0.302$, $r=0.449$, $p=0.0809$. The solid line is a calculated regression line, and the two dashed lines show 95% confidence intervals. In the above equations, y is the concentration of 1,2-CHdiol-U, 1,4-CHdiol-U or CHol-U (in mg/g creatinine), and x is the concentration of CH-A (in ppm). r is a correlation coefficient (dimensionless). $n=16$.

Urinary concentrations of CHol and CHdiols

Urinary concentrations were expressed as observed and creatinine (cr)-corrected values. The concentrations of CHdiols-U and CHol-U are summarized in Table 1. Nine samples of 1,2-CHdiol-U and 10 samples of 1,4-CHdiol-U at baseline were below the LOQ, while all end-of-shift urine samples were higher than the LOQ. All baseline CHol-U samples and 12 of the end-of-shift CHol-U samples were below the LOQ. The GMs of the cr-corrected concentrations of 1,2-CHdiol-U, 1,4-CHdiol-U and CHol-U at the baseline were 1/17, 1/15 and 1/1.3 of those collected at the end of the working week, respectively. The end-of-shift concentrations of CHdiols-U were significantly higher than those at baseline ($p<0.0001$), but no difference was observed in CHol-U.

Correlation between the personal exposure concentration of CH and urinary concentrations of CHdiols and CHol

Scatter diagrams with calculated regression lines and 95% confidence intervals for CHdiols-U and CHol-U are depicted in Fig. 2. A simple regression analysis indicated that both CHdiols-U at the end of the shift were significantly correlated with CH-A ($p<0.0001$). The correlation coefficients for 1,2-CHdiol-U and 1,4-CHdiol-U were 0.852 and 0.847, respectively. No correlation was observed between CH-A and CHol-U ($r=0.449$, $p=0.0809$).

Discussion

Our study showed that end-of-shift CHdiols-U were significantly correlated with CH-A at exposure levels lower than half of the OEL proposed by the JSOH. We also observed no correlation between CH-A and CHol-U. There are a few field studies on biological monitoring of CH^{9, 10, 16, 17}, and the four biomarkers that they evaluated were urinary unmetabolized CH (CH-U), CHone (CHone-U), CHdiols and CHol.

CH-U is the most specific of the four biomarkers, because CHdiols-U, CHol-U and CHone-U can also be metabolites of CHol and CHone, whereas CH-U cannot (Fig. 1)⁷. Ghittori *et al.*⁹ reported a high correlation coefficient ($r=0.89$) between CH-A and CH-U. However, the practical use of CH-U is limited because CH-U requires on-site sample preparation in order to minimize loss or contamination. Yasugi *et al.*¹⁰ reported that CHone-U and CHol-U were significantly correlated with CH-A; however, CHone-U concentrations were lower than 1/10 of CHol-U concentrations, and the correlation between CHone-U and CH-A was weak. Therefore, CH-U and CHone-U were excluded from our investigation.

The DFG proposed using 1,2-CHdiol-U for the BAT because it is not a normal constituent of human

urine⁷). However, it can appear in urine due to nonoccupational exposure because CH is detected in the indoor environment of houses in general^{7,18}, as it is a component of washings, glues and adhesives. In this study, the concentration of metabolites in baseline urine can be regarded as a background level in unexposed subjects (i.e., controls) because the baseline urine sample was collected after a 5 day-holiday, that is, with no CH exposure for 120 hours. Perico *et al.*¹⁶ reported that background levels (GM) in subjects without occupational exposure to organic solvents were 0.4 mg/g cr for 1,2-CHdiol-U and 1.2 mg/g cr for 1,4-CHdiol-U, respectively. Kawai *et al.*¹⁹ reported that no CHol or 1,4-CHdiol was found in any urine samples and that the background level (GM) of 1,2-CHdiol was 0.99 mg/g cr. These results are consistent with our results.

CHdiols-U accumulate in the body during the working week because the elimination half-lives ($t_{1/2}$) of 1,2-CHdiol and 1,4-CHdiol are 17.0 ± 5.2 (hours) and 16.1 ± 3.9 (hours), respectively¹¹. This means that the regression equation using data collected on the first working day of the week is different from that collected on the last working day, even in the same subjects exposed to a constant concentration of CH. Perico *et al.*¹⁶ reported that end-of-shift CHdiols-U on Monday (the first working day of the week) were significantly correlated with CH-A and that their correlation coefficients were larger than those of end-of-shift CHdiols-U on Thursday. They calculated regression equations using data collected on Monday but did not do the same on Thursday. In contrast, we obtained regression equations using data collected at the end of the shift on Friday (the last day of five consecutive work days) but not on Monday. Therefore, it is difficult to directly compare the regression equations in these two studies. To overcome this problem, we applied a pharmacokinetic approach that the DFG used to reevaluate the BAT of CH¹². According to the DFG approach, the accumulation factors (AF) calculated from the following formula were 1.59 for 1,2-CHdiol and 1.54 for 1,4-CHdiol, respectively.

$$AF = [1 - \exp(-n \times k_{el} \times \tau)] / [1 - \exp(-k_{el} \times \tau)]$$

k_{el} is a first-order elimination rate constant (calculated as $k_{el} = \ln(2) / t_{1/2}$), τ is the exposure interval (24 hours), and n is the number of exposures (5 times). This means that after the last shift on the last day of five consecutive work days at a constant exposure level, the concentrations of 1,2-CHdiol-U and 1,4-CHdiol-U increased to 1.59 times and 1.54 times respectively, compared with after only one day of exposure. The regression equation reported by Perico *et al.*¹⁶ was y (mg/g creatinine) = $0.081x$ (mg/m³) + 3.9 ($r=0.6662$, $p<0.001$), where y is 1,2-CHdiol-U and x is CH-A, or $y=0.2835x$ (ppm) + 3.9, because 1 ppm = 3.5 mg/m³

of CH. The corresponding equation in this study is y (mg/g creatinine) = $0.466x$ (ppm) + 3.354 ($r=0.852$, $p<0.0001$). The slope of the regression equation in this study is 1.64 times larger than the one reported by Perico *et al.*, which is in agreement with the accumulation factor for 1,2-CHdiol (1.59) calculated above. As in the case of 1,2-CHdiol, the ratio of the slopes of the two regression equations for 1,4-CHdiol is 1.56, and this is also in agreement with its calculated accumulation factor (1.54). Therefore, these results suggest that the two regression equations are essentially the same. From the calculated regression equation in Fig. 2, the concentrations of 1,2-CHdiol-U after 8 hours exposure to 1/10 and 1/2 the TLV-TWA proposed by the JSOH were as follows: 10.3 mg/g cr with a 95% confidence limit of 7.7–13.0 mg/g cr and 38.3 mg/g cr with a 95% confidence limit of 29.5–47.2 mg/g cr, respectively.

In the two previous studies, CHol-U was significantly correlated with CH-A ($r=0.811$ and 0.795 after enzymatic hydrolysis, without correction for urine density)^{10,17}, but this was not the case in this study. One possible reason for this may be differences in exposure level, because the amount of CHol excreted into the urine is a very small proportion (<1%) of the CH absorbed¹⁰. The personal exposure concentrations of CH in the studies of Yasugi *et al.*¹⁰ and Perbellini *et al.*¹⁷ were 27 ppm as GM (274 ppm as the maximum) and 17–2,484 mg/m³ as the range (or 4.9–709.7 ppm), respectively, whereas the corresponding value in this study was 18.1 ppm as GM (4.5–60.3 ppm as the range). Other crucial factors may be differences in the time of sample collection and/or differences in determination methods. These suggest that CHol-U may correlate closely with CH-A at a high level of exposure but not necessarily so at a low level of exposure such as the range investigated in this study. Mraz *et al.*²⁰ studied the effect of ethanol ingestion on the urinary excretion of CHol and CHdiols using volunteers. They reported that the elimination of CHol was substantially enhanced by ingestion of 56 g of ethanol during exposure. In this study, though personal exposure monitoring of ethanol was not conducted, the GM concentration of ethanol in workplace air by stationary sampling was 4 ppm. Uptake of ethanol inhaled by our subjects during 8 hours was estimated to be approximately 47 mg, with two assumptions (lung adsorption rate, 0.62; respiratory volume, 10 m³)²¹, and this level was far less than that of the experiments by Mraz *et al.* Therefore, the enhancement effect seemed to be negligible in our subjects.

In conclusion, CHdiols-U at the end of the last shift of the working week are considered suitable biomarkers of occupational exposure to CH, while CHol-U is

not a suitable biomarker at low levels of exposure.

Acknowledgments: This work was supported in part by a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan.

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