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Monitoring of occupational exposure to cyclohexanone by diffusive sampling and urinalysis

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The present study was initiated in order to identify the best marker of occupational exposure to cyclohexanone among cyclohexanone and its metabolites in urine. To examine if diffusive samplers are applicable to personal monitoring of exposure to cyclohexanone in workroom air, the performance of carbon cloth to adsorb cyclohexanone in air was studied by experimental exposure of the cloth to cyclohexanone at 5, 10, 25 or 50 ppm (i.e. 20, 40, 100 or 200 mg m^{-3}) for up to 8 h. Cyclohexanone in the exposed cloth was extracted with carbon disulphide followed by gas chromatographic (GC) analysis. The cloth adsorbed cyclohexanone in proportion to the concentration (up to 50 ppm) and the duration (up to 8 h), and responded quantitatively to a 15 min exposure at 100 ppm. In a field survey, end-of-shift urine samples were collected from 24 factory workers occupationally exposed to cyclohexanone (up to 9 ppm) in combination with toluene and other solvents. Urine samples were also collected from 10 subjects with no occupational exposure to solvents. The urine samples were treated with acid or an enzyme preparation for hydrolysis, and extracted with dichloromethane or ethyl acetate. The extracts were analysed by GC for cyclohexanone, cyclohexanol, and *trans*- and *cis*-isomers of 1,2- and 1,4-cyclohexanediol. Both cyclohexanol and *trans*-1,2-cyclohexanediol in urine correlated significantly with time-weighted average intensity of exposure to cyclohexanone. Although *trans*-1,4-isomer was also excreted, its quantitative relation with cyclohexanone exposure could not be established, because the solvent extraction rate was low and unstable. Excretion of *cis*-isomers was not confirmed. The two analytes, cyclohexanol and *trans*-1,2-cyclohexanediol, appeared to be equally valid as exposure markers, but the latter may be superior to the former in the sense that it is sensitive enough to separate the exposed from the non-exposed at 1 ppm or less cyclohexanone exposure.

Keywords: biological monitoring, carbon cloth, cyclohexanone, *trans*-1,2-cyclohexanediol, cyclohexanol, diffusive sampling, urinalysis.

Abbreviations: CHone, cyclohexanone; CHol, cyclohexanol; CHdiol, cyclohexanediol (e.g. *trans*-1,2-CHdiol for *trans*-1,2-cyclohexanediol).

Introduction

Cyclohexanone (molecular weight, 98.14; CHone) is an alicyclic ketone with a freezing point of –32.1 °C and a boiling point of 155.6 °C (Verschueren 1983). A paper has been published recently to report neurotoxic effects of this solvent among occupationally exposed workers (Mitran *et al.* 1997). Although a minor component, this slow vaporizing ketone has been frequently detected in industrial solvent preparations, especially those for printing (Ukai *et al.* 1997), indicating a potential need of biological monitoring of exposure.

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Existing reports on its metabolism *in vivo*, however, suggest that biotransformation in humans may be different from that in experimental animals. For example, cyclohexanediol (CHdiol; dihydroxyl metabolites, especially 1,2-isomer) is a major metabolite in urine samples from babies who are exposed to this solvent during the course of an infusion support (Mills and Walker 1990) and those from human volunteers exposed experimentally to CHone (Mraz *et al.* 1994a,b). Subsequently, successful applications in occupational health showed that CHol is a valid indicator of occupational exposure to this solvent (Ong *et al.* 1991a,b). In contrast, cyclohexanol (CHol; a mono-hydroxyl metabolite) is a leading metabolite detected in the serum of rabbits (Sakata *et al.* 1993) and in the urine of dogs (Martis *et al.* 1980) after experimental administration. Thus, which one of the mono- and di-hydroxyl metabolite(s) would be an analyte of choice for biological monitoring of factory workers exposed to this popular alicyclic ketone remains to be investigated.

In this study, gas chromatographic (GC) methods were developed to measure mono- and di-hydroxylated CHone (i.e. CHol and CHdiol) in urine, and the methods were applied to urine samples obtained from CHone-exposed factory workers to evaluate CHdiol in comparison with CHone and CHol as an indicator of occupational exposure to CHone.

Subjects, materials and methods

Exposure of carbon cloth to cyclohexanone, and gas chromatographic analysis

Carbon cloth (KF-1500) employed for diffusive sampling (Hirayama and Ikeda 1979, Ikeda *et al.* 1984) was a product from Toyobo, Osaka, Japan. In an experimental exposure study to examine the ability of the cloth to adsorb CHone, the cloth was exposed to CHone vapour utilizing a servo-mechanized system for solvent vapour exposed (Koizumi and Ikeda 1981). The difference between the ordered concentration and the mean observed concentration (as automatically monitored by GC every 5 min) was less than 3 % of the ordered concentration and the coefficient of variation of the observed concentration was 5 % or less. The vapour concentration range was selected by taking the current occupational exposure limit into consideration [i.e. 25 ppm as a time-weighted average concentration by both the American Conference of Governmental Industrial Hygienists (1997) and the Japan Society for Occupational Health (1997)]. An additional exposure was made for 15 min at 100 ppm to stimulate a short-term peak exposure.

Factories and workers in the study

The survey was conducted in two small-scale lacquer-ware production plants, and all workers in the plants (12 men and 12 women, i.e. 24 subjects in total) volunteered to participate in the survey. The analysis of the workroom air by diffusive sampling (by the method described below) followed by GC analysis showed that the workers were exposed to CHone in addition to other solvents, as summarized in table 1; CHone was the second most common solvent in the workroom air, next to toluene. Each worker was equipped with one diffusive sampler on the cloth at a chest pocket level from 10:00 am to 05:00 pm on a Thursday. At the end of the period, the sampler was collected and each worker offered his/her urine sample in a solvent vapour-free room. A walk-through survey disclosed that the workers did not wear protective hand gloves, but none of them had skin contact with liquid lacquer material under usual operating conditions. For comparison, Thursday afternoon urine samples were collected from six men and four women working in the business sections with no occupational exposure to solvent.

Analysis of exposed carbon cloth

Analysis of the exposed carbon cloth for CHone was carried out by carbon disulphide (CS_2) extraction followed by GC analysis (with a flame ionization detector; or FID-GC) as previously described (Yasugi *et al.* 1994). The GC analysis conditions are summarized in table 2, and a typical chromatogram is shown in figure 1[A]. Under the conditions described, the detection limit (when a peak/noise ratio of 2 was taken) was 0.1 ng per injection, which is roughly equivalent to the amount of CHone detectable in the cloth after exposure to 0.01 ppm CHone for 8 h. Although the peak for CHone

Table 1. Concentrations of solvent vapours detected in breathing zone air of the workers

Solvent	Vapour concentration[a]		
	GM	GSD	Max.
Cyclohexanone	0.86	5.54	9.3
Toluene	2.31	5.03	19.1
Xylenes[b]	0.71	4.19	11.3
Ethyl acetate	0.33	4.00	4.2
Ethylbenzene	0.81	3.77	7.7
Ethyleneglycol monoethyl ether	0.21	2.45	1.0
Ethyleneglycol monobutyl ether	0.25	3.32	2.1

[a] Eight-hour time-weighted average concentrations; GM for geometric mean (in ppm), GSD for geometric standard deviation (dimensionless), and Max. for the maximum concentration observed (ppm).
[b] Three xylene isomers in combination, mostly *m*-isomer.

Table 2. Gas chromatographic analysis conditions

Item	Carbon cloth analysis	Urinalysis	
		Condition 1	Condition 2
Analyte[a]	CHone	CHone and CHol	CHdiols
Sample	CS_2 extract (1 µl)	DCM extract (1 µl)	EA extract (1 µl)
Autosampler	HP[b] Model 7673	HP Model 7673A	HP Model 7673A
Column	DB-WAX[c] 60 m × ϕ0.25 mm (f. th.[d] 0.5 µm)	DB-1[c] 30 m × ϕ0.53 mm (f. th.[d] 1.5 µm)	DB-1[c]
Column temperature	42 °C for 9 min, 6 °C min^{-1} to 60 °C 10 °C min^{-1} to 80 °C 80 °C for 15 min, 15 °C min^{-1} to 185 °C	40 °C for 7 min, 5 °C min^{-1} to 100 °C 40 °C min^{-1} to 200 °C 200 °C for 10 min	80 °C for 3 min, 5 °C min^{-1} to 120 °C 40 °C min^{-1} to 200 °C 200 °C for 10 min
Injection port temperature	200 °C	250 °C	250 °C
Detector temperature	210 °C	250 °C	250 °C
Retention time (min)	CHone 29.4	CHone 13.4 CHol 14.6	*trans*-1,2-CHdiol 9.3 *cis*-1,2-CHdiol 9.4 *trans*-1,4-CHdiol 10.4 *cis*-1,4-CHdiol 10.6
Time for a run (min)[e]	36	31.5	23

A gas chromatograph, HP 5890 Series II with a flame ionization detector, was used in all cases He as carrier gas and make-up gas was allowed to flow at 1.9 ml min^{-1} and 40 ml min^{-1}, respectively. The supply of H_2 and air to the detector were at 40 ml min^{-1} and 450 ml min^{-1}, respectively.
[a] CHone, cyclohexanone; CHol, cyclohexanol; CHdiol, cyclohexanediol.
[b] HP, Hewlett-Packard, Philadelphia PA, USA.
[c] DP-WAX and DB-1, products of J & W, Folsom CA, USA.
[d] f. th., film thickness.
[e] Excluding the time for cooling down the column.

took rather a long time to appear (i.e. at 29.4 min), the coefficient of variation after quadruplicate analyses of 10 µg CHone per ml CS_2 was as small as 0.6 %, indicating validity of the measurements despite this long retention time.

Urinalysis for cyclohexanone, cyclohexanol and cyclohexanediol

Urine samples were analysed under three different pretreatment conditions of (a) enzymatic hydrolysis (which was used as a standard procedure, as discussed later), (b) acid hydrolysis and (c) no hydrolysis, as developed for the determination of 2,5-hexanedione after *n*-hexane exposure (Kawai *et al.* 1990). The details of the three pretreatment procedures have also been described previously (Kawai *et al.* 1990).

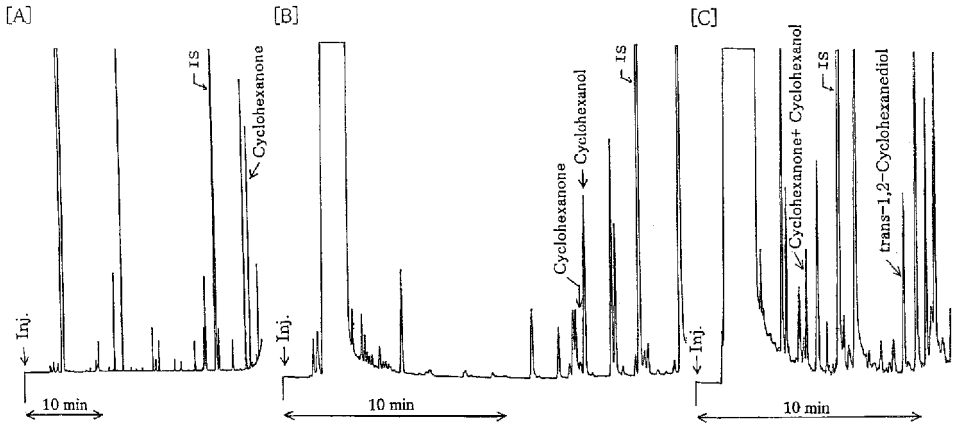

Figure 1. Typical gas chromatograms to show isolation of [A] cyclohexanone in CS_2 extract (24.6 µg cyclohexanone ml^{-1} CS_2) on a DB-WAX column, [B] cyclohexanone [0.055 mg l^{-1} urine (0.035 mg l^{-1} when corrected for a specific gravity of 1.016)] and cyclohexanol [0.932 mg l^{-1})] in DCM extract on a DB-1 column under GC Condition 1, and [C] *trans*-1,2-cyclohexanediol [10.3 mg l^{-1} urine (7.2 mg l^{-1})] in EA extract on a DB-1 column under Condition 2. In the cases of [B] and [C], the material analysed was an end-of-shift urine sample collected from a worker exposed to cyclohexanone at 7.5 ppm and 2.8 ppm, respectively (both as a time-weighted average). The internal standard (IS) was isobutylbenzene (202.7 µg ml^{-1} CS_2 extract) in the case of Chromatogram [A], and 3-methylcyclohexanone (17.9 µg ml^{-1} ethyl acetate extract) in cases of Chromatograms [B] and [C]. For details of analytical conditions and retention times, see table 2.

In a standard assay with enzymic hydrolysis, an aliquot (5 ml) of a urine sample was placed into a screw-capped 10-ml Pyrex glass tube (with a Teflon septum), and mixed with 0.5 ml of 1 M acetate buffer (pH 4.7) and 1 ml of the enzyme solution (for details of the enzyme solution, see the Reagents section). After tight sealing of the tube the mixture was incubated at 37 °C for 24 h, and then extracted with either 2 ml dichloromethane (DCM) or ethyl acetate (EA; both spiked with 17.9 µg 3-methylhexanone ml^{-1}) by vigorous shaking for 1 min. The tube was spun at 1940 × *g* for separation of the organic solvent phase from water. The DCM or EA extract, 1 µl per injection, was introduced into a GC.

In the case of acid hydrolysis, 5 ml urine was mixed with 0.25 ml 35 % hydrochloric acid in a screw-capped 10-ml Pyrex tube (with a Teflon septum), and the tube, after tight sealing, was heated in a boiling water bath for 30 min per hydrolysis. The acid-hydrolysed sample was extracted in the same way as described for enzymic pretreatment. In the case of no hydrolysis, the urine sample was extracted without any pretreatment.

GC conditions for the analysis of the DCM or EA extract are summarized in table 2. Two separate column temperature conditions were employed for urinalaysis to cope with the slow appearance of CHdiol on the chromatogram, i.e. Condition 1 for CHone and CHol (i.e. the low temperature condition), and Condition 2 for 1,2- and 1,4-CHdiol (i.e. the high temperature condition). Under these analytical conditions, the detection limits were 0.01 mg per litre urine for both CHone and CHol. The coefficient of variation (CV) was 1.5 % and 1.1 % when either 2.47 mg of CHone or CHol per litre water was analysed five times, respectively.

In the cases of 1,2- and 1,4-CHdiol, the detection limits were 0.1 mg l^{-1} for the 1,2-isomer and 1.0 mg l^{-1} for the 1,4-isomer (i.e. the peak area in the chromatogram for the 1,4-isomer was approximately 14 % of that of the 1,2-isomer when compared on an equi-molar basis). After five analyses of solutions containing 100 mg per litre of water the CV were 2.0 % and 7.1 % for the 1,2-isomer and the 1,4-isomer, respectively. Thus, both the detection limit and the CV were greater for CHdiols than for CHone and CHol among the analytes, and greater for the 1,4-isomer than for the 1,2-isomer among the two CHdiol isomers. Typical chromatograms are shown in figure 1([B] and [C]). In a preliminary study, CHol in 34 urine samples (from 24 exposed and 10 non-exposed workers) was measured under Condition 1 and also under Condition 2. Comparison by paired *t*-test showed no significant ($p > 0.10$) difference between the results of the two measurements, suggesting that the analysis results under both conditions are quantitatively equivalent.

Three metabolites of CHol, *trans*-1,2-CHdiol and *trans*-1,4-CHdiol were subjected to identification by gas chromatography–mass spectrometry (GC–MS). The GS–MS used was a combination of a gas chromatograph (Hewlett Packard 5890) and a mass spectrometer (Hewlett Packard 5970B) equipped with a DB-1 column (J & W; 50 m, 0.32 mm and 1.0 µm in length, inner diameter and film thickness) and a

reference library (Hewlett Packard Chemi-station software G1034CLJ). When an ethyl acetate solution of the reference chemicals and an ethyl acetate extract of a urine sample (from a worker exposed to CHone at 8 ppm) were analysed, total ion chromatography (TIC) gave exactly the same retention times for each of CHol, *trans*-1,2-CHdiol and *trans*-1,4-CHdiol in the two samples. Comparison of the spectra gave an agreement rate of 81 % for CHol, 87 % for *trans*-1,2-CHdiol and 50 % for *trans*-1,4-CHdiol; the low agreement rate for *trans*-1,4-CHdiol was probably due to the low rate of extraction with ethyl acetate.

In some instances, the analyte concentrations were expressed after correction for urine density in terms of creatinine concentration (Jackson 1966) or a specific gravity of 1.016 (Rainsford and Davies 1965). Creatinine concentration and specific gravity were measured by colorimetry and refractometry, respectively.

Reagents

CHone, CHol, CS_2, DCM, EA and 3-methylhexanone were purchased from Kanto Chemicals, Tokyo, Japan. Steric isomers of CHdiol (*trans*-1,2-CHdiol, *cis*-1-2-CHdiol, and a mixture of *cis*- and *trans*-1,4-CHdiol) were purchased from Aldrich Chemicals, Milwaukee WIS, USA.

Statistical analysis

Urinary concentrations on a group basis were expressed in terms of a geometric mean (GM) and a geometric standard deviation (GSD) together with the number of determinations (N) with an assumption of log-normal distribution. Linear regression analysis, and Student's t-test (both paired and unpaired) were employed as necessary.

Results

Ability of carbon cloth to adsorb cyclohexanone

Utilizing a vapour exposure system (Koizumi and Ikeda 1981), three pieces each of carbon cloth (housed in holder cases individually) were exposed to CHone vapours at 0, 5, 10, 25 or 50 ppm (0, 20, 40, 100 or 200 mg m^{-3}, respectively) for 1, 2, 4, or 8 h, and the amount of CHone adsorbed was determined by GC analysis after extraction with CS_2. Plotting of the amount of CHone against the length of exposure or concentration showed that the amount adsorbed was linearly related up to the longest time of exposure (8 h) as well as up to the highest exposure concentration (50 ppm) tested. Thus, it was possible to express the amount of CHone adsorbed on the carbon cloth as

$$Z = 5.26 \times X \times Y$$

where Z is the amount of CHone (in μg) in the exposed cloth, X is the exposure duration (h) and Y is the exposure concentration (ppm). The parameter, 5.26, was experimentally obtained.

In order to examine the ability of the cloth to respond to a short-term peak CHone exposure, five pieces of carbon cloth were exposed for 15 min (or 0.25 hr) at 100 ppm. When the amount of CHone detected in the cloth was compared with the amount estimated by the equation cited above, the average amount detected (130 μg in the exposed cloth) was 98.9 % of the estimate (131.5 μg in the cloth exposed for 15 min). Thus, it was concluded that the carbon cloth can adsorb CHone quantitatively even under the condition of a short-term (e.g. 15 min) peak (e.g. 100 ppm) exposure.

In a separate experiment to examine possible decay due to spontaneous desorption, carbon cloth was exposed to CHone at 50 ppm for 2 h. The exposed cloth was kept in a holder and left in fresh air for up to 48 h after the termination of the exposure. The amounts detected in the exposed cloth at any time after the

initiation of the fresh air exposure did not differ from the amounts detected immediately after the termination of exposure, which indicates that no spontaneous desorption of adsorbed CHone takes place from the exposed carbon cloth.

Choice of organic solvent for extraction of cyclohexanone and its metabolites in urine

It was previously determined that DCM can extract both CHone and CHol from urine (Yasugi *et al.* 1994) and it was also reported that EA can be employed for extraction of CHone metabolites (Mraz *et al.* 1994a). It was further suspected that the rate of extraction of hydroxylated CHone metabolites (especially CHdiol) from water (or urine) to the organic solvents may not be high due to their hydrophilic nature. To examine if such would be the case, CHol (101 mg l^{-1}), *trans*-1,2-CHdiol (102 mg l^{-1}) and a mixture of *cis*- and *trans*-1,4-CHdiol (74 mg l^{-1} in combination) were dissolved together into DCM or EA, and 2 ml of the DCM or EA solution was shaken in a tube (to achieve an equilibrium between the organic solvent and the water) with 5 ml of water, or water added with the acid (to simulate the acid hydrolysis condition) or with the buffer and the enzyme solution (to simulate the enzymic hydrolysis condition). When the tube was spun for separation, it was shown that the two phases were more readily separated when shaken with DCM than with EA. A portion of the organic phase thus obtained was subjected to GC analysis.

From the results summarized in table 3, it is clear that both CHone and CHol were extracted well into both DCM and EA, whereas the extraction rate was substantially lower for CHdiol, especially for 1,4-CHdiol. When extracted with DCM, not only was the rate below 1 %, but the coefficient of variation in the extraction rate was 50 % (in the case of acid hydrolysis) or even greater (i.e. 64 % in the case of enzymic hydrolysis). The rate for 1,4-CHdiol extraction was improved by several times when EA was employed, although the rate was still as low as 2 % or less. On GC analysis, the resolution of the CHol peak from the CHone peak was hardly achieved when GC Condition 2 for urinalysis (table 2) was employed. Thus, it was considered best to apply the DCM extract to GC under Condition 1 for determination of CHone and CHol, and to use the EA extract to analyse four CHdiol isomers under GC Condition 2. For quantification of CHone and its metabolites in urine, corrections were made for different extraction rates among the analytes.

Levels of cyclohexanone, cyclohexanol and cyclohexanediol in urine from non-exposed subjects

In order to investigate background levels of the CHone metabolites, the three pretreatment procedures for hydrolysis coupled with two extraction and GC conditions thus established were applied first to urine samples collected from six men and four women who were not occupationally exposed to any solvents. The results of analyses are summarized in the top half of table 4. Because no sex difference could be detected ($p > 0.10$), the results from men and women are combined in the table. It was previously known that no CHone was detected in urine samples from non-exposed subjects (Yasugi *et al.* 1994). Similarly, no CHol was found in any urine samples after acid hydrolysis or no hydrolysis, and only minute excretion of CHol was detected after enzymic hydrolysis. In contrast, *trans*-1,2-CHdiol was detected at measurable concentrations in all the 10 subjects (six men and four women) after acid hydrolysis and two cases (two men and no women) after enzymic hydrolysis. The concentrations of *cis*-1,2-CHdiol, *trans*-1,4-CHdiol or *cis*-1,4-CHdiol was below the detection limit.

Table 3. Extraction rate of cyclohexanone and its metabolites from urine to dichloromethane or ethyl acetate

Pretreatment Solvent[b]	CHone[a] (101 mg l^{-1})[c]	CHol[a] (101 mg l^{-1})[c]	1,2-CHdiol[a] (102 mg l^{-1})[c]	1,4-CHdiol[a] (74 mg l^{-1})[c]
No hydrolysis				
DCM[d]	98.4 (0.3%)	71.6 (3.6%)	2.3 (3.7%)	0.5 (16.0%)
EA[d]	83.2 (1.8%)	79.2 (0.2%)	6.1 (4.6%)	2.2 (13.8%)
Acid hydrolysis				
DCM[d]	97.9 (0.9%)	71.2 (0.2%)	2.2 (1.4%)	0.4 (54.3%)
EA[d]	86.2 (1.6%)	78.2 (0.5%)	7.7 (7.5%)	2.2 (16.9%)
Enzymic hydrolysis				
DCM[d]	98.4 (0.8%)	71.1 (0.5%)	2.7 (8.5%)	0.6 (64.3%)
EA[d]	86.3 (0.7%)	76.0 (0.5%)	6.1 (2.3%)	1.5 (4.8%)

Values are mean recovery rates in % followed by the coefficients of variation of the recovery in parentheses.

[a] CHone, cyclohexanone; CHol, cyclohexanol; 1,2-CHdiol, *trans*-1,2-cyclohexanediol; 1,4-CHdiol, a mixture of *trans*- and *cis*-1,4-cyclohexanediol.

[b] Solvent used for equilibrium experiment.

[c] Concentration added to the organic solvent.

[d] DCM, dichloromethane; EA, ethyl acetate.

Table 4. Excretion of cyclohexanol and *trans*-1,2-cyclohexanediol in urine of non-exposed subjects

Analyte Pretreatment	Correction for urine density None	 Creatinine	 Sp.Gr(1.016)
Non-exposed (10 subjects; 6 men and 4 women in combination)			
CHol			
Acid hydrolysis	ND	ND	ND
Enzymic hydrolysis	0.03 (1.77)	0.04 (2.01)	0.03 (1.87)
Trans-1,2-CHdiol			
Acid hydrolysis	0.75 (1.82)	0.99 (2.73)	1.23 (2.41)
Enzymic hydrolysis	0.13 (1.69)	0.18 (2.12)	0.16 (1.83)
Exposed (24 subjects; 12 men and 12 women in combination)			
CHol			
Acid hydrolysis	0.17 (7.00)	0.19 (7.18)	0.16 (6.84)
Enzymic hydrolysis	0.08 (4.61)	0.09 (4.86)	0.08 (4.51)
Trans-1,2-CHdiol			
Acid hydrolysis	7.31 (2.35)	8.79 (2.32)	6.79 (2.31)
Enzymic hydrolysis	4.13 (2.75)	4.97 (2.76)	3.84 (2.74)

Geometric mean (GM) and geometric standard deviation (GSD) are shown. The unit for GM is mg l^{-1} when corrected for none (i.e. as observed) or for a specific gravity of 1.016, or mg (g creatinine)$^{-1}$ when corrected for creatinine concentration. When no analyte was detected in urine, the concentration was assumed to be at the detection limit of 0.01 mg l^{-1} [or 0.01 mg (g creatinine)$^{-1}$] for CHol, and 0.1 mg l^{-1} [or 0.1 mg (g creatinie)$^{-1}$] for *trans*-1,2-CHdiol in calculating GM and GSD.

Lack of (or very minute) excretion of CHone or its metabolites in unconjugated forms

In order to examine if CHone or its metabolites are excreted in urine even as unconjugated, three urine samples each from exposed (at 2.5, 7.5 and 7.9 ppm CHone as time-weighted averages) and non-exposed workers (all men) were subjected to the three types of pretreatment, and analysed for CHone and CHol under Condition 1 and for CHdiols under Condition 2. A small but measurable amount of *trans*-1,2-CHdiol (about 1 mg l^{-1} or less) was detected in urine from the non-exposed subjects after acid or enzymic hydrolysis, but not so when no hydrolysis pretreatment was applied, and no CHone, CHol or 1,4-CHdiol was

detected with or without hydrolysis in confirmation with what was described above. With regard to the urine samples from the exposed, only small quantities of CHone and CHol (both about 0.01 mg l^{-1}) and no 1,4-CHdiol were detected when no hydrolysis was applied, although the level of 1,2-CHdiol was measurable (i.e. up to 5 mg l^{-1}). With hydrolysis, however, the levels of 1,2-CHdiol detected were much higher, e.g. 24 mg l^{-1} after enzymic hydrolysis. Accordingly, further quantitative analysis was made only after hydrolysis.

Increased levels of cyclohexanol and trans-1,2-cyclohexanediol in urine among the workers occupationally exposed to cyclohexanone vapour

The quantitative relationship between the time-weighted average concentration of CHone in breathing zone air and the level of CHone, CHol or CHdiol isomers was examined by regression analysis. Preliminary analyses with men and women separated showed that there was no sex difference in the slopes and the vertical axis intercepts ($p > 0.10$) of the regression lines in the exposure–excretion relationship. Accordingly, the data of the two sexes (both exposed and non-exposed) were combined for statistical analysis, the results of which are summarized in the bottom half of table 4 and in table 5.

The average concentrations of the two metabolites, CHol and *trans*-1,2-CHdiol, are given in the bottom half of table 4. Thus, about 0.1 mg CHol and 4 mg *trans*-1,2-CHdiol per litre urine were detected as geometric means in urine samples from workers exposed to 0.9 ppm CHone; the wide variation in exposure intensity was reflected by large geometric standard deviation.

The excretion of CHone in urine was very minute, i.e. the slope (β) was essentially zero, independent of acid or enzymic hydrolysis of urine samples. Although the slope (β) was steeper when urine samples were hydrolysed with enzymes (e.g. 0.004 mg l^{-1} ppm^{-1} for the non-corrected case) than when they were pretreated with acid (e.g. < 0.001 mg l^{-1} ppm^{-1}), and the *r* value (0.775) was statistically significant ($p < 0.01$) after enzymic hydrolysis, the dose-dependent increment in urinary CHone was too small to be considered for biological monitoring of CHone exposure.

The increase in CHol in urine was dependent on the intensity of CHone exposure (table 5). The *r* values were all statistically significant ($p < 0.01$) after acid or enzymic hydrolysis independent of the correction for urine density and the slope (β) after acid hydrolysis (0.15–0.19 mg l^{-1} [or (g creatinine)$^{-1}$] ppm^{-1}) was twice as steep as that after enzymic hydrolysis (0.08–0.09 mg l^{-1} [or (g creatinine)$^{-1}$] ppm^{-1}). Nevertheless, *r* values after acid hydrolysis (0.75–0.81) were smaller than the values after enzymic hydrolysis (0.89–0.92) under all three conditions of correction for urine density. Scatter diagrams after enzymic hydrolysis and correction for a specific gravity of 1.016 are depicted in figure 2[A].

Trans-1,2-CHdiol in urine also showed dose-dependent increases when urine samples were pretreated for hydrolysis (figure 2[B]). The slopes (β), 1.2–2.3 mg l^{-1} [or (g creatinine)$^{-1}$] ppm^{-1}, were more than 10 times greater than those for CHol excretion {0.08–0.19 mg l^{-1} [or (g creatinine)$^{-1}$] ppm^{-1}} even when the differences in molecular weights were taken into account. The *r* values for *trans*-1,2-CHdiol [0.65–0.82; being statistically significant ($p < 0.01$) after either hydrolysis] were essentially the same after acid or enzymic hydrolysis, and comparable to the counterpart coefficients for CHol (0.75–0.92).

Table 5. Cyclohexanone exposure-dependent increase in cyclohexanol, *trans*-1,2-cyclohexanediol and trans-1,4-cyclohexanediol in urine of workers

Analyte in urine Pretreatment of urine Correction for urine density[a]	Regression line parameter[b] α	β	*r*
Cyclohexanol			
Acid hydrolysis			
None (mg l^{-1})	0.092	0.185	0.753**
Creatinine [mg (g creatinine)$^{-1}$]	0.126	0.169	0.810**
Specific gravity (mg l^{-1})	0.096	0.151	0.801**
Enzyme hydrolysis			
None (mg l^{-1})	0.000	0.091	0.887**
Creatinine [mg (g creatinine)$^{-1}$]	0.017	0.088	0.899**
Specific gravity (mg l^{-1})	0.007	0.077	0.923**
Trans-1,2-cyclohexanediol			
Acid hydrolysis			
None (mg l^{-1})	1.16	1.50	0.784**
Creatinine [mg (g creatinine)$^{-1}$]	1.83	1.39	0.727**
Specific gravity (mg l^{-1})	1.27	1.23	0.820**
Enzymic hydrolysis			
None (mg l^{-1})	1.26	2.31	0.653**
Creatinine [mg (g creatinine)$^{-1}$]	2.07	1.88	0.720**
Specific gravity (mg l^{-1})	1.38	1.78	0.729**
Trans-1,4-cyclohexanediol			
Acid hydrolysis			
None (mg l^{-1})	52.83	7.82	0.095
Creatinine [mg (g creatinine)$^{-1}$]	66.11	15.13	0.129
Specific gravity (mg l^{-1})	48.07	9.73	0.128]
Enzymic hydrolysis			
None (mg l^{-1})	–6.77	11.29	0.546**
Creatinine [mg (g creatinine)$^{-1}$]	–3.76	7.13	0.624**
Specific gravity (mg l^{-1})	–4.43	7.70	0.581**

[a] Analyte concentration in urine were corrected for either none (i.e. as observed), creatinine concentration, or a specific gravity of 1.016.

[b] α and β are parameters of a regression line (calculated with 24 exposed and 10 non-exposed cases, men and women in combination). of $Y = \alpha + \beta X$, where X is the time-weighted average cyclohexanone exposure concentration (in ppm) and Y is the analyte concentration in urine (in unit as shown in the table). r is a correlation coefficient, and asterisks show statistical significance (** and * for $p < 0.01$ and 0.05, respectively) of the coefficient.

Excretion of *trans*-1,4-CHdiol was nominally more abundant than its 1,2-isomer as the steep slopes {7 mg l^{-1} [or (g creatinine)$^{-1}$] ppm^{-1} or even greater} showed. The results, however, should be treated with caution because r values were generally small, being insignificant after acid hydrolysis. Such small r values (or wide variation) may be due at least in part to the low and unstable extraction rate (table 3) of 1,4-CHdiol from water or urine to EA.

Discussion

The present study demonstrated that diffusive sampling is applicable to personal ambient air monitoring in occupational settings (figure 1). Taking advantage of the diffusive sampling in combination with the GC methods established, it was further made clear that *trans*-1,2-CHdiol and most probably *trans*-1,4-CHdiol (but no *cis*-isomers of both CHdiols) are excreted in urine, dependent on the intensity of CHone exposure, and that *trans*-1,2-CHdiol can be

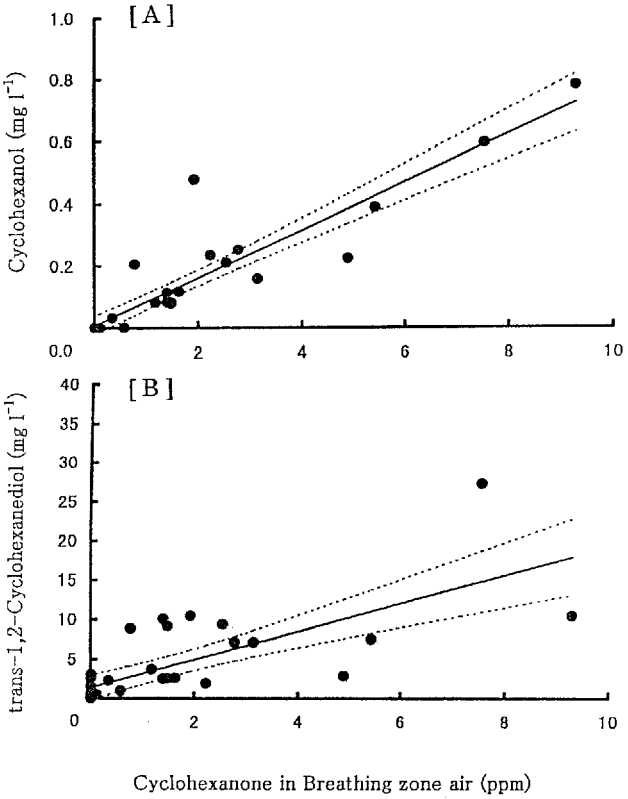

Figure 2. Relationship between the exposure to cyclohexanone vapour and excretion of [A] cyclohexanol and [B] *trans*-1,2-cyclohexanediol in urine. The analyte levels were after enzymic analysis and correction for a specific gravity of urine of 1.016. The line in the middle is a calculated regression line (for equations, see table 5), and the curves on both sides of the line show the 95% confidence ranges of the regression line. Each dot shows one case.

used, in addition to CHol, as a urinary marker of exposure to CHone. The dose-dependent increase in excretion of CHone in urine was very small.

In the GC analysis system developed for the determination of CHone, CHol and CHdiol, the major problem is the low extraction rate for di-hydroxylated metabolites into the organic solvents. The low extraction rate may be inherent to the hydroxyl moiety, and was more evident with 1,4-CHdiol (table 3). Thus, it is not yet possible to establish a solid quantitative relationship between CHone in breathing zone air and 1,4-CHdiol in urine, although the urinary excretion of 1,4-CHdiol after exposure to CHone is most likely. The choice of DCM for extraction was in principle based on the analogy of the case of 2,5-hexanedione as a hexane metabolite (Kawai *et al.* 1990), and this solvent was also used in cyclohexane biomonitoring (Yasugi *et al.* 1994). It was also considered at first that it may not be practical in occupational health to employ various extractants to target analytes, because pretreatment (including solvent extraction) rather than GC analysis is the most time-consuming process of the determination, especially when an automated liquid sampler is available for GC.

The extraction rate of DCM was low for CHdiols, even though the rate was almost constant for CHol and 1,2-CHdiol irrespective of the various urine samples.

EA, originally used by Mraz *et al.* (1994a), gave an extraction rate which was several times higher than that given by DCM when applied to 1,4-CHdiol extraction. Nevertheless, the rate of 2% or less for 1,4-CHdiol is far from satisfactory and the rate is not always reproducible. Another problem is poor separation of the EA phase from the water phase even after centrifugation. It is thus necessary to find an organic solvent which can extract CHdiols from urine at a high extraction rate and which is easy to separate from water. With regard to the choice between acid and enzymic hydrolysis for pretreatment of urine, enzymic hydrolysis is recommended because enzymic hydrolysis gives fewer peaks on the chromatogram than acid hydrolysis (the peaks might confound the quantification of target analytes, as was the case for cyclohexane metabolites) (Yasugi *et al.* 1994).

Selection of the target analyte(s) for biological monitoring of CHone exposure is worthy of discussion. Williams (1959) described that when administered to rabbits CHone gave rise to glucuronized CHol in urine. Later, Martis *et al.* (1980) detected CHol in the plasma of beagle dogs given CHone i.v., and Sakata *et al.* (1993) measured time course of the CHol level in the plasma of rabbits after oral administration of CHone. Subsequently, Ong *et al.* (1991a,b) determined CHol in urine of workers occupationally exposed to CHone at 1–40 ppm (Ong *et al.* 1991a) or 2–30 ppm (Ong *et al.* 1991b) and found a dose-dependent increase in the end-of-shift urine samples with correlation coefficients of 0.66–0.73 (depending on the correction for urine density; Ong *et al.* 1991a) or 0.88 (Ong *et al.* 1991b).

In an extension of clinical observation on the babies who were supported by infusion systems, Mills and Walker (1990) detected CHdiols in the urine of the babies. The most abundant isomer was *trans*-1,2-CHdiol; 1,3- and 1,4-isomers were present in small quantities and *cis*-1,2-isomer was present in trace quantities. According to the authors, the probable source of CHone was as a contaminant of the infusion liquid, and also by leaching from the infusion set. Mraz *et al.* (1994a,b) exposed human volunteers to CHone at up to 100 ppm for 8 h and analysed urine samples for CHol and CHdiols. 1,2-CHdiol was excreted most abundantly (39% of the estimated CHone dose absorbed in the respiratory tract) followed by 1,4-CHdiol (18%) and then CHol (1%). Based on the observation, they recommended CHdiols rather than CHol as biological markers of occupational exposure to CHone. The longer half-life of CHdiols (16–18 h; Mraz *et al.* 1994b) in urinary excretion compared with that of CHol (less than 2 h; Mraz *et a*. 1994b) is also advantageous.

In the present study, the quantitative comparison between CHone, CHol and CHdiols in urine showed that the level of *trans*-1,2-CHdiol was more than 10 times as high as CHol, and that very little CHone was detected. No *cis*-1,2-CHdiol was detected. The findings on the excretion of *trans*-1,4-CHdiol and the lack of *cis*-1,4-CHdiol excretion should be taken as tentative and not conclusive. The difficulty was at least partly due to technical problems such as low extraction rates even with EA and low sensitivity of the FID to both isomers of 1,4-CHdiols, as discussed above.

The present survey showed that 0.091 mg CHol l^{-1} ppm^{-1} and 2.31 mg *trans*-1,2-CHdiol l^{-1} ppm^{-1} will be excreted in the end-of-shift urine after occupational exposure to CHone (the values in table 5, after enzymic hydrolysis and without correction for urine density). With two assumptions that a worker is exposed to CHone at the occupational exposure limit of 25 ppm or 100 mg m^{-3} [American Conference of Governmental Industrial Hygienists (1977) and Japan Society for

Industrial Health (1996)], and that the subject excretes urine at 1 ml min^{-1} (Yasugi *et al.* 1994), the urinary excretion of CHol and CHdiol at the end of a day-shift will be 0.0227 μmole CHol min^{-1} and 0.497 μmole CHdiol min^{-1} [namely (0.091 mg l^{-1} min^{-1} 100.16^{-1} for CHol, and 2.31 mg l^{-1} min^{-1} 116.16^{-1} for CHdiol) × 25 (ppm) × 10^{-3} ml l^{-1}, respectively], when the moleculars weights of 100.16 for CHol and 116.16 for CHdiol are taken into account.

With further three assumptions that the subject inhales 10 litres (or 10^{-2} m^3) min^{-1} of workroom air (Yasugi *et al.* 1994), that the uptake ratio of CHone in the lungs of humans is 58 % (Mraz *et al.* 1994a) and that skin absorption is only a minor route of CHone absorption (Mraz *et al.* 1994a) and can be ignored under the working conditions, the subject, when exposed at 25 ppm (or 100 mg m^{-3}) will absorb 5.91 μmoles CHone min^{-1} [100×10^{-3} (g m^{-3}) × 98.14^{-1} (moles g^{-1}) × 0.58×10^{-2} (m^3 min^{-1})]. Thus, about 8.8 % of the CHone absorbed will be excreted into urine as CHol (0.4 %) and *trans*-1,2-CHdiol (8.4 %), of which CHol is a minor metabolite (accounting for about 4 % of the sum of the two metabolites) and *trans*-1,2-CHdiol a major one (96 %). Urinary excretion of CHone in the form of other metabolites such as 1,4-CHdiol should also be taken into account, and their quantitative role in excretion kinetics apparently needs to be elucidated. Such quantitative evaluation should be taken as preliminary data, however, because the exposure of the workers studied was rather low (i.e. less than 10 ppm in contrast to the current occupational exposure limit of 25 ppm as discussed above) and only two cases were available in the range of 6–10 ppm.

When CHol and *trans*-1,2-CHdiol were compared on the basis of the exposure of marker of choice from the viewpoint of regression analysis, correlation coefficients were similar for the two analytes (table 5). Thus, further comparative evaluation of the sensitivity of CHol and *trans*-1,2-CHdiol is warranted. Two approaches were previously presented (Kawai *et al.* 1992a,b), taking (as a check point) the lowest solvent concentration in breathing zone air at which the urinary marker of exposure can statistically separate the exposed from the non-exposed (the lowest separation concentration, or LSC). The LSC can be set as the solvent concentration in air (ppm) at which the lower 95 % confidence limit for the urinary maker is equal to the upper 95 % confidence limit at 0 ppm (LSC_1). In cases where the marker is known to be present even in the urine of non-exposed subjects (i.e. background level excretion), the LSC can also be calculated as the solvent

Table 6. Lowest concentration of cyclohexanone to separate the exposed from the non-exposed when cyclohexanol and 1,2-cyclohexanediol are used as markers of cyclohexanone exposure

Analyte in urine Correction for urine density	Lowest Separation Conc. (LSC)[a]	
	LSC_1[a]	LSC_2[a]
Cyclohexanol		
None (mg l^{-1})	1.0 ppm	1.4 ppm
Creatinine [mg (g creatinine)$^{-1}$]	0.9 ppm	1.9 ppm
Specific gravity (mg l^{-1})	0.8 ppm	1.6 ppm
Trans-1,2-cyclohexanediol		
None (mg l^{-1})	2.1 ppm	0.6 ppm
Creatinine [mg (g creatinine)$^{-1}$]	1.8 ppm	0.3 ppm
Specific gravity (mg l^{-1})	1.6 ppm	0.4 ppm

[a] The lowest separation concentration (LSC) is the solvent concentration in air at which the exposure marker in the biological material (e.g. urine) can statistically separate the exposed from the non-exposed. For further definition of LSC, LSC_1 and LSC_2, see the Discussion section.

concentration at which the lower 95 % confidence limit for the urinary maker is equal to the upper 95 % confidence limit of the background level (LSC_2).

When such approaches were applied to the cases of CHol and *trans*-1,2-CHdiol after enzymic hydrolysis (table 6), it was found that CHol can separate the exposed from the non-exposed at 1 ppm CHone when the LSC_1 approach was taken, and it was at 1.4–2 ppm with the LSC_2 approach. In the case of *trans*-1,2-CHdiol, LSC_1 was about 2 ppm and LSC_2 was about 0.5 ppm. Thus, both CHol and *trans*-1,2-CHdiol may be equally sensitive, being effective at 2 ppm CHone, or *trans*-1,2-CHdiol may be more sensitive than CHol when the LSC_2 approach is taken.

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