

Thioether excretion in urine of applicators exposed to 1,3-dichloropropene: a comparison with urinary mercapturic acid excretion

R T H van Welie, C M van Marrewijk, F A de Wolff, N P E Vermeulen

Abstract

The excretion of thioethers in urine of applicators occupationally exposed to the soil fumigant 1,3-dichloropropene (DCP) was determined by the thioether assay. The mercapturic acid metabolite of E-1,3-dichloropropene, N-acetyl-S-(E-3-chloropropenyl-2-)-L-cysteine (E-DCP-MA), was the reference compound in the thioether assay. The mean recovery of E-DCP-MA was 58.5% (coefficient of variation (CV) 9%, n = 4). In non-exposed men mean background of urinary thioethers was 6.05 mmol SH/mol creatinine (n = 56). In applicators exposed to soil fumigants containing DCP, urinary excretion of thioethers followed first order elimination kinetics. Urinary half lives of elimination of thioethers were 8.0 (SD 2.5) hours based on excretion rates and 9.5 (SD 3.1) hours based on creatinine excretion. The urinary half life of elimination of thioethers was almost twofold higher compared with half lives of elimination of the mercapturic acids of Z- and E-1,3-dichloropropene. The post- minus pre-shift thioether concentrations in urine and the cumulative urinary thioether excretions correlated well with exposure to DCP. In urine samples the mean thioether concentration was 1.38 higher than mean DCP mercapturic acid concentration. This suggests the presence of unidentified thioether metabolite(s) due to exposure to soil fumigants containing DCP. According to the present data, an eight hour time weighted average exposure to the Dutch occupational exposure limit of 5 mg/m³ DCP

results in a post- minus pre-shift thioether concentration of 9.6 mmol SH/mol creatinine (95% confidence interval (95% CI) 7.4-11.8 mmol SH/mol creatinine) and in a cumulative thioether excretion of 139 μ mol SH (95% CI 120-157 μ mol SH). It is concluded that the thioether assay can be used to assess comparatively high levels of exposure to DCP.

Exposure of man to electrophilic compounds may ultimately lead to the excretion of thioethers such as cysteine conjugates and mercapturic acids (S-substituted N-acetyl-L-cysteine conjugates) in urine. Mercapturic acids are end products from metabolic detoxication of electrophilic chemicals via conjugation to glutathione. Glutathione conjugation either proceeds spontaneously or is catalysed by glutathione S-transferases present in liver, blood, and several other organs and tissues. Once glutathione conjugates have been formed in vivo, metabolic cleavage of the glutamyl and glycine residue generally takes place. N-acetylation of the remaining S-alkylated cysteine conjugates finally leads to the formation of the mercapturic acid.

Mercapturic acids excreted in urine can be determined as thioethers using the thioether assay. The determination of urinary thioethers can be used as a non-selective test to assess exposure to electrophilic compounds.¹ The thioether assay has been used frequently to compare exposed and non-exposed populations, for example, factories producing rubber,² pharmaceuticals and explosives, or upon exposure to bitumen fumes.^{3,4} Mostly, however, no information is available about the chemical structure of the various thioethers determined. Besides a limited selectivity, the sensitivity of the thioether assay is also limited by background excretion of thioethers. This is strongly influenced by lifestyle factors (for instance, smoking and medication). By contrast, for the determination of mercapturic acids in urine, selective and sensitive analytical techniques such as high performance liquid chromatography and gas chromatography with different detection techniques are available.^{5,6}

Recently, occupational exposure to Z- and E-1,3-

Department of Pharmacochemistry, Division of Molecular Toxicology, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

R T H van Welie, N P E Vermeulen

Department of Human Toxicology, University Hospital of Leiden, Leiden, The Netherlands

C M van Marrewijk, F A de Wolff

dichloropropene (Z- and E-DCP) of applicators in the Dutch flower bulb culture was determined and shown to result in urinary excretion of two mercapturic acids—namely, N-acetyl-S-(Z- and E-3-chloropropenyl-2-)-L-cysteine (Z- and E-DCP-MA). A linear relation between exposure to Z- and E-DCP and excretion of Z- and E-DCP-MA in urine was found. Urinary half lives of elimination were 5.0 (SD 1.2) hours for Z-DCP-MA and 4.7 (SD 1.3) hours for E-DCP-MA.⁷

The primary aim of our study was to determine the relation between exposure to soil fumigants containing DCP and urinary excretion of thioethers in occupationally exposed applicators. The post- minus pre-shift thioether concentrations and cumulative thioether excretion were correlated with exposure to DCP. Half lives of elimination of thioethers in urine were also determined. Finally, excretion of thioether was compared with urinary mercapturic acid excretion.⁷

Materials and methods

CHEMICALS

N-acetyl-S-(Z- and E-3-chloropropenyl-2-)-L-cysteine were synthesised.⁸ Potassium dihydrogen phosphate and citric acid (trisodium salt dihydrate) were purchased from Merck (Darmstadt, Germany). 5,5'-Dithiobis-2-nitrobenzoic acid and creatinine stock solutions were obtained from Sigma (St Louis, MO, USA). N-acetyl-L-cysteine was purchased from Janssen Chimica (Beerse, Belgium).

POPULATION AND EXPOSURE

The population under investigation consisted of 12 male Dutch applicators of Z- and E-DCP in the flower bulb culture and four male non-exposed volunteers. The respiratory exposure to Z- and E-DCP of these applicators has been the subject of a combined biological, environmental, and biological effect monitoring study.^{7,9,10} Population characteristics, application techniques, and sampling strategies of urine and air samples have been described extensively in these papers. Briefly, the eight hour time weighted average (TWA) exposures to Z- and E-DCP were determined by means of personal air sampling during full period consecutive sampling. The exposure to (Z + E)-DCP ranged from 0.3 to 18.9 mg/m³ in shifts of one to 11 hours. The urinary excretion of the corresponding mercapturic acids, Z- and E-DCP-MA, was determined in samples collected before, during, and after the exposure measurements. Mean urinary half lives of elimination were 5.0 (SD 1.2) hours for Z-DCP-MA and 4.7 (SD 1.3) hours for E-DCP-MA. Maximum cumulative urinary excretion of (Z + E)-DCP-MA amounted to 296 µmol. Strong correlations ($r \geq 0.93$) between exposure to Z- and E-DCP and the excre-

tion of respective mercapturic acids in urine were found.⁷

ANALYSIS OF THIOETHERS

The urinary excretion of thioethers was determined as described previously with slight modifications.¹¹ Samples of human urine were centrifuged for five minutes at 4000 g and 2 ml portions were transferred into glass stoppered tubes. The pH was adjusted to 1.5–2.0 with 0.25 ml 4 M hydrochloric acid. Two × 4 ml of ethyl acetate were used for extraction of the acidified urine samples by vortex mixing for 30 seconds. The organic layers were separated by centrifugation for five minutes at 4000 g and evaporated to dryness at 37°C under a mild nitrogen flow. The residues obtained were resuspended in 1 ml distilled water. Subsequently alkaline hydrolysis was performed on 0.5 ml portions in screw capped glass tubes by adding 0.25 ml 4 M sodium hydroxide, saturating with nitrogen, and heating at 100°C in a boiling water bath in the dark. After hydrolysing for 50 minutes, samples were cooled on ice for 10 minutes and 0.25 ml of 4 M hydrochloric acid was added. To the remaining, non-hydrolysed portions of 0.5 ml of resuspended residue, 0.5 ml 4 M sodium chloride was added.

After exactly five minutes, the sulphhydryl (SH) concentrations in the hydrolysed and non-hydrolysed samples were determined by adding Ellman reagent and measuring the absorbance at 412 nm. Freshly prepared Ellman reagent consisted of 1.0 ml 1.0 M phosphate buffer (pH 7.1) and 0.3 ml 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) solution (0.4 mg DTNB per ml 1% sodium citrate).¹²

Standard calibration curves of SH groups were prepared by dissolving N-acetyl-L-cysteine in concentrations ranging from 0.10 to 0.80 mmol/l in 2 M sodium chloride and directly analysing with Ellman reagent. Calibration curves of thioethers were prepared by dissolving N-acetyl-S-(E-3-chloropropenyl-2-)-L-cysteine (E-DCP-MA) in concentrations ranging from 0.10 to 0.80 mmol/l in human urine, and treating as described above. Both types of calibration curve were prepared fresh daily.

Urinary thioether concentrations were calculated using differences in absorbance at 412 nm between the hydrolysed and the non-hydrolysed parts of the samples in the regression equation of the calibration curve of E-DCP-MA. The recovery of E-DCP-MA was calculated from the ratios of the absorbance at 412 nm of E-DCP-MA *v* the absorbance at 412 nm of the equimolar SH concentrations of N-acetyl-L-cysteine.

ANALYSIS OF CREATININE

Urinary creatinine was determined by the Jaffe reaction.¹³

Table 1 Concentration dependent within day and day to day variation in recovery of E-DCP-MA in thioether assay

E-DCP-MA (mmol/l)	Within day			Day to day		
	Recovery (%)	CV (%)	No	Recovery (%)	CV (%)	No
0.10	52.7	2.5	3	58.8	28.5	25
0.20	58.5	6.6	3	60.9	17.8	25
0.40	65.5	2.3	3	65.1	11.7	25
0.53	—*	—	—	70.2	9.3	14
0.80	57.1	3.9	3	50.5	6.4	11
Mean	58.5	9.0		61.1	12.0	

*Not determined.

CALCULATIONS

Urinary elimination rate constants (k_{el}) of the thioethers were calculated by linear regression analysis from the linear parts of the semilogarithmic thioether excretion rate v time curves or from the thioether excretion expressed per mol creatinine v time curves. Elimination rate constants were used to calculate half lives of elimination ($t_{1/2} = \ln 2/k_{el}$). Correlation coefficients (r) were calculated as a measure for the strength of linear relations. Spearman correlations (ρ) were calculated to test for the significance of the correlation coefficients. Levels of significance between urinary half lives of elimination of mercapturic acids of DCP and thioethers were assessed by the two tailed Wilcoxon signed rank test.

Results

ANALYSIS OF THIOETHERS

Both types of standard calibration curves, N-acetyl-L-cysteine in 2 M sodium chloride and E-DCP-MA in human urine, showed linear relations ($r \geq 0.998$; $n = 25$). Table 1 presents within day and day to day recoveries of E-DCP-MA determined with the thioether assay. The mean within day recovery of E-DCP-MA in concentrations ranging from 0.10 to 0.80 mmol/l was 58.5% (coefficient of variation (CV) = 9%, $n = 4$). The CV was $\leq 6.6\%$ for all E-DCP-MA concentrations tested. Mean day to day recovery was 61.1% (CV = 12.0%, $n = 5$). Mean within day and mean day to day recoveries were not significantly different. The day to day CV increased

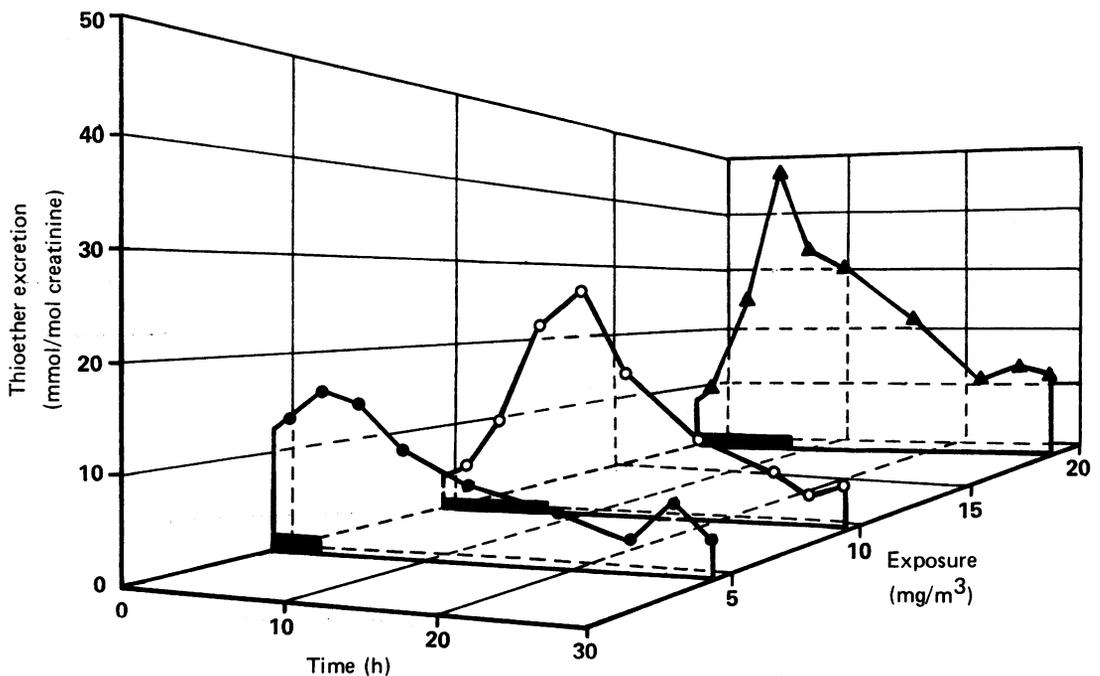


Figure 1 Urinary excretion of thioethers (per mol creatinine) of applicators exposed to 3.8 (●), 9.8 (○), and 18.9 (▲) mg/m³ eight hour TWA (Z + E)-DCP in respiratory air. Dark shaded areas indicate exposure periods.

Table 2 Urinary half lives of elimination of thioethers and mercapturic acids of (Z + E)-DCP based on excretion rates (mg/h) and on creatinine excretion (mmol/mol creatinine) in applicators occupationally exposed to soil fumigants containing DCP

	$t_{1/2}$ of elimination (h (SD))	
	Mercapturic acids†	Thioethers
Excretion rate (n = 5)	4.6 (1.0)	8.0 (2.5)*
Creatinine excretion (n = 9)	4.9 (0.8)	9.5 (3.1)**

* $p < 0.05$; ** $p < 0.01$,
†Taken from reference².

with decreasing concentrations of E-DCP-MA, reaching a value of 28.5% at 0.10 mmol/l. The high values of the day to day CVs were probably due to variation in the efficiency of the alkaline hydrolysis step in the thioether assay. From day to day the efficiency of the alkaline hydrolysis varied from 60 to 90%. The efficiency of the ethyl acetate extraction of E-DCP-MA was 94.7 (SD 6.6)%; Z-DCP-MA appeared to have the same recovery in the thioether assay as E-DCP-MA. Therefore, only E-DCP-MA was selected as a reference compound for this study.

BACKGROUND THIOETHER CONCENTRATIONS

Control urine samples were obtained from four volunteers (n = 27) and 12 applicators (n = 29). Background thioether concentrations were log normally distributed over a range from 1.26 to 16.33 mmol SH/mol creatinine. The mean background was 6.05 mmol SH/mol creatinine with a 95% confidence interval (95% CI) lower limit of 5.48 mmol SH/mol creatinine and an upper limit of 6.68 mmol SH/mol creatinine.

URINARY THIOETHER EXCRETION

Figure 1 shows the urinary thioether excretion profiles expressed per mol creatinine, of three applicators, exposed to 3.8, 9.8, and 18.9 mg/m³ eight hours time weighted average (TWA) (Z + E)-DCP respectively. Pre-shift thioether concentrations corresponded with background concentrations of thioethers seen in control urine samples. Thioether excretion increased rapidly after exposure to DCP started and thioether concentrations appeared to be dependent on duration and level of exposure. Maximum thioether concentrations measured at the exposures presented in fig 1 were 16.6, 27.3, and 48.0 mmol SH/mol creatinine respectively.

Post-shift urinary excretion of thioethers followed first order elimination kinetics. Urinary elimination rate constants were calculated if the correlation coefficients of the semilog linear parts of the excretion *v* time curves were $r \geq 0.917$ and if at least four post-shift data points with SH concentrations higher than the background excretion levels, observed in con-

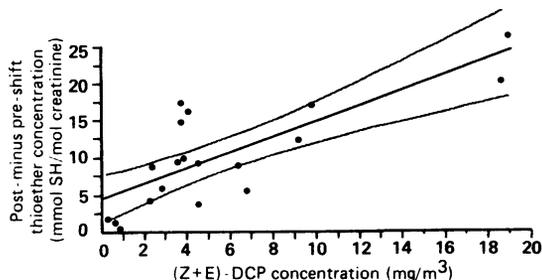


Figure 2 Correlation between eight hour TWA (Z + E)-DCP concentration in respiratory air and urinary post-minus pre-shift thioether concentrations in applicators. Regression line $y = 4.37 + 1.05x$ ($r = 0.79$; $n = 19$; $p = 0.001$) and 95% CIs are drawn.

trols, were available. Urinary elimination rate constants were used to calculate $t_{1/2}$ of elimination. Only a few excretion profiles met all these requirements, primarily due to background excretion of thioethers. The $t_{1/2}$ of elimination was calculated both from excretion rates and creatinine excretion. Based on excretion rates, half lives of elimination were 9.8, 8.1, and 9.7 hours, respectively (fig 1). Mean $t_{1/2}$ of elimination based on excretion rates was 8.0 (SD 2.5) hours (n = 5) and based on creatinine excretion 9.5 (SD 3.1) hours (n = 9). A roughly twofold larger statistically significant difference ($p < 0.05$) in $t_{1/2}$ of elimination of thioethers was seen when compared with the urinary $t_{1/2}$ of elimination of mercapturic acids determined previously (table 2).

THIOETHER EXCRETION IN RELATION TO DCP EXPOSURE

Figure 2 shows the relation between the eight hour TWA exposure to (Z + E)-DCP and the thioether concentration, expressed per mol creatinine, of the first urine sample collected post-shift minus the thioether concentration of the sample collected pre-shift. Subtraction of the pre-shift thioether concentration served as an individual correction of the

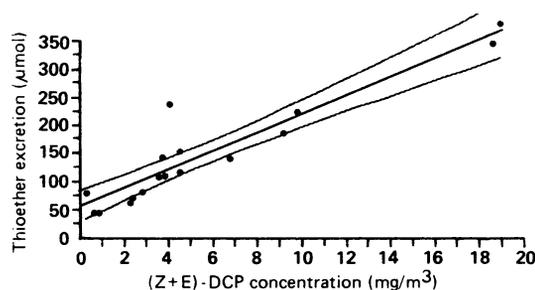


Figure 3 Correlation between eight hour TWA (Z + E)-DCP concentration in respiratory air and cumulative urinary thioether excretion in applicators. Regression line $y = 56.5 + 16.5x$ ($r = 0.93$; $n = 17$; $p < 0.001$) and 95% CIs are drawn.

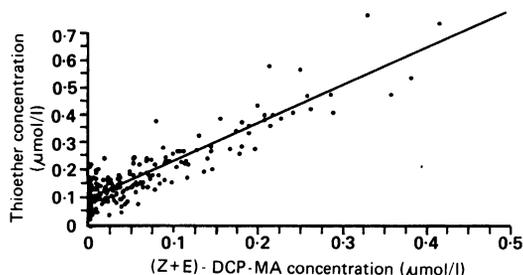


Figure 4 Correlation between (Z + E) DCP-MA concentration and thioether concentration in urine samples of applicators. Regression line $y = 0.09 + 1.38x$ ($r = 0.90$; $n = 221$; $p < 0.001$) is drawn.

background thioether excretion and improved the correlation with exposure to DCP. The correlation coefficient between DCP exposure and post- minus pre-shift thioether concentration was $r = 0.79$.

Figure 3 shows the relation between the eight hour TWA exposure to (Z + E)-DCP and the cumulative thioether excretion in urine. Cumulative excretion of thioethers was calculated as the area under the urinary thioether excretion rate v time curve (AUC) from the start of the shift until eight hours post-shift. Data for two applicators were omitted; one applicator wore a respirator and the excretion curve of the other was too irregular to interpret. A correlation coefficient of $r = 0.93$ was found between exposure to DCP and cumulative excretion of thioethers in urine.

COMPARISON OF THIOETHER AND MERCAPTURIC ACID EXCRETION

In a previous study,⁷ concentrations of Z- and E-DCP-MA were determined in the same urine samples in which the presently reported thioether concentrations were measured. Figure 4 shows the relation between (Z + E)-DCP-MA concentrations and the thioether concentrations. Urine samples in which no mercapturic acids were detected and samples in which the thioether concentration was in the

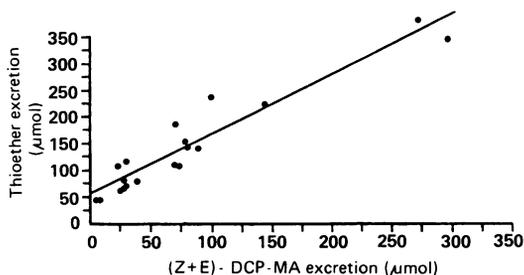


Figure 5 Correlation between cumulative urinary (Z + E)-DCP-MA excretion and cumulative urinary thioether excretion. Regression line $y = 56.48 + 1.11x$ ($r = 0.95$; $n = 19$; $p < 0.001$) is drawn.

range of the background level (≤ 6.68 mmol SH/mol creatinine), were omitted from the regression analysis. The regression line was $y = 0.09 + 1.38x$ ($r = 0.90$).

Figure 5 compares the cumulative urinary excretion of (Z + E)-DCP-MA and of thioethers, both calculated as the area under the excretion rate v time curves, for 19 shifts. Cumulative excretion of the thioethers was calculated from the start of the shift until eight hours post-shift. Cumulative excretion of mercapturic acid was also calculated from the start of shift. Terminal excretion, however, was calculated by extrapolating time to infinity using the elimination rate constant.⁷ The regression line was $y = 56.48 + 1.11x$ ($r = 0.95$).

Discussion

In this study, the excretion of thioethers in urine of applicators of DCP was investigated in relation to the exposure to this soil fumigant. Urinary half lives of elimination of thioethers were calculated, and thioether and mercapturic acid excretion were compared. Also, the thioether assay was evaluated quantitatively.

At present, no data concerning extraction recoveries and detection limits of thioethers in the thioether assay are available. N-acetyl-S-(E-3-chloropropenyl-2-)-L-cysteine (E-DCP-MA), a mercapturic acid metabolite of E-DCP, was therefore used as a synthetic standard to evaluate the analytical performance of the thioether assay. Standard calibration curves of SH groups and calibration curves of thioethers in human urine all showed good linearity. The recovery of E-DCP-MA from human urine was only 58.5% (CV = 9%, $n = 4$) but did not differ significantly day to day. The within day recovery CV remained below 6.6% for E-DCP-MA concentrations ranging from 0.10 to 0.80 mmol/l urine. Day to day recovery CV of E-DCP-MA, however, amounted to 28.5% at 0.10 mmol/l E-DCP-MA, stressing the need for preparing calibration curves daily. The detection limit of E-DCP-MA in the thioether assay was 0.10 mmol/l. Lower concentrations could be detected as thioether but the CV exceeded 10%. Previously, recoveries of Z- and E-DCP-MA from urine were found to be 69% (CV = 7%, $n = 4$) and 70% (CV = 8%, $n = 4$) when measured by gas chromatography. With this technique, detection limits of Z- and E-DCP-MA were 0.45 $\mu\text{mol/l}$ (CV = 7.2%, $n = 8$).⁵

In urine samples obtained from non-exposed volunteers and applicators, a mean thioether background of 6.05 mmol SH/mol creatinine was found. Comparable background concentrations ranging from 3.8 to 5.1 mmol SH/mol creatinine were found previously in other reference populations.^{4,11} Smoking, medication, and other confounding factors

of lifestyle were not taken into account in our present study. None of the subjects was taking medicines, however, and only four of 12 applicators were moderate smokers. Moderate smoking did not result in significantly increased urinary excretion of thioether in the group of non-exposed volunteers.

The shapes of the urinary thioether excretion profiles appeared to be similar at different exposures in the applicators. As already found with Z- and E-DCP-MA excretion, thioether excretion rapidly increased after exposure to DCP started and followed first order excretion kinetics. The height of thioether excretion depended on the level and duration of exposure. Pre-exposure and, in general, more than eight hours post-exposure, normal background excretion concentrations of thioethers were seen in applicators.

Urinary $t_{1/2}$ values for elimination of thioethers were 8.0 (SD 2.5) hours and 9.5 (SD 3.1) hours based on excretion rates or on creatinine excretions, respectively. Compared with the $t_{1/2}$ of elimination of the mercapturic acids of DCP, $t_{1/2}$ of elimination of thioethers was almost twice as high. This significant difference can be explained by the presence of a (DCP) thioether metabolite(s) with a longer $t_{1/2}$ of elimination than those of (Z + E)-DCP-MA. Exposure to impurities in commercial DCP formulations could be the reason for differences between urinary thioether excretion and urinary mercapturic acid excretion.

Two urinary thioether excretion parameters—namely, post- minus pre-shift thioether concentration and cumulative thioether excretion—proved to be successful in assessing exposure to DCP. Both parameters showed good linear relations with exposure to DCP. Cumulative Z- and E-DCP-MA excretion also correlated well with exposure to DCP.⁷ In line with both findings, thioether and mercapturic acid concentrations in urine samples of applicators showed a strong linear relation. The intercept of the regression equation corresponded to thioether concentrations found in blank urine samples. The slope of 1.38 supported the postulated presence of an unidentified thioether metabolite(s). Cumulative thioether excretion also exceeded cumulative mercapturic acid excretion. In calculating the first, however, no corrections were made for the background excretion and in calculating the second, excretion to infinity was taken into consideration.

The question remains at which DCP exposures the mercapturic acid and thioether assay can be applied successfully. The determination of the mercapturic acids of DCP by gas chromatography with different detection techniques proved to be much more sensitive and selective than the determination of thioethers with the thioether assay. Moreover, the mercapturic acids of DCP were not detectable in

urine of subjects not exposed to this soil fumigant. Exposure to <0.09 mg/m³ (eight hour TWA) for 4.9 hours, however, resulted in detectable Z-DCP-MA concentrations in urine of volunteers. By contrast, thioethers are excreted with background concentrations depending on lifestyle. The upper limits of the 95% CI of the intercepts of the post- minus pre-shift concentrations (7.5 mmol SH/mol creatinine) and the cumulative excretion (88 μ mol SH) indicate the increments in thioether excretion that are necessary to distinguish between samples from exposed and non-exposed subjects. According to the respective regression equations 7.5 mmol SH/mol creatinine corresponded to an eight hour TWA exposure of 3.0 mg/m³ DCP and 88 μ mol SH to an eight hour TWA exposure of 1.9 mg/m³ DCP. The thioether assay can be used to assess comparatively high DCP exposure levels.

An eight hour TWA exposure to the Dutch occupational exposure limit of 5 mg/m³ DCP would result in a post- minus pre-shift thioether concentration of 9.6 mmol SH/mol creatinine with a 95% CI of 7.4 to 11.8 mmol SH/mol creatinine. The same exposure level would result in an excretion of 139 μ mol SH with a 95% CI of 120 to 157 μ mol SH. These values could be regarded as warning limits to be used by occupational hygienists.

This investigation was financially supported by the Directorate General of Labour of the Ministry of Social Affairs and Employment and the Ministry of Housing, Physical Planning, and the Environment.

Requests for reprints to: N P E Vermeulen, Department of Pharmacochemistry, Division of Molecular Toxicology, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

- 1 Doorn van R, Leijdekkers Ch-M, Bos RP, Brouns RME, Henderson PTh. Detection of human exposure to electrophilic compounds by assay of thioether detoxication products in urine. *Ann Occup Hyg* 1981;24:77-92.
- 2 Kilpikari I. Correlation of urinary thioethers with chemical exposure in a rubber plant. *Br J Ind Med* 1981;38:98-100.
- 3 Ahlborg G, Bergström B, Högstedt C, Einistö P, Sorsa M. Urinary screening for potentially genotoxic exposures in a chemical industry. *Br J Ind Med* 1985;42:691-9.
- 4 Burgaz S, Bayhan A, Karakaya AE. Thioether excretion of workers exposed to bitumen fumes. *Int Arch Occup Environ Health* 1988;60:347-9.
- 5 Welie van RTH, Duyn van P, Vermeulen NPE. Determination of two mercapturic acid metabolites of 1,3-dichloropropene in human urine with gas chromatography and sulphur-selective detection. *J Chrom Biomed Appl* 1989;496:463-71.
- 6 Vermeulen NPE. Analysis of mercapturic acids as a tool in biotransformation, biomonitoring and toxicological studies. *Tips* 1989;10:177-81.
- 7 Welie van RTH, Duyn van P, Brouwer DH, Hemmen van JJ, Brouwer EJ, Vermeulen NPE. Inhalation exposure to 1,3-dichloropropene in the Dutch flower-bulb culture. Part II. Biological monitoring by measurement of urinary excretion of two mercapturic acid metabolites. *Arch Environ Contam Toxicol* 1991;20:6-12.

- 8 Onkenhout W, Mulder PPJ, Boogaard PJ, Buijs W, Vermeulen NPE. Identification and quantitative determination of mercapturic acids formed from Z- and E-1,3-dichloropropene by the rat, using gas chromatography with three different detection techniques. *Arch Toxicol* 1986;59:235-41.
- 9 Brouwer DH, Brouwer EJ, Vreede de JAF, Welie van RTH, Vermeulen NPE, Hemmen van JJ. Inhalation exposure to 1,3-dichloropropene in the Dutch flower-bulb culture. Part I. Environmental monitoring. *Arch Environ Contam Toxicol* 1991;20:1-5.
- 10 Brouwer EJ, Evelo CIA, Verplanke AJW, Welie van RTH, Wolff de FA. Biological effect monitoring of occupational exposure to 1,3-dichloropropene: effects on liver and renal function and on glutathione conjugation. *Br J Ind Med* 1991;48:167-72.
- 11 Doorn van R, Leijdekkers Ch-M, Bos RP, Brouns RME, Henderson PTh. Enhanced excretion of thioethers in urine of operators of chemical waste incinerators. *Br J Ind Med* 1981;38:187-90.
- 12 Ellman GI. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;82:70-7.
- 13 Tietz NW. *Textbook of clinical chemistry*. Philadelphia: WB Saunders, 1986:1279.

Accepted 26 November 1990

Vancouver style

All manuscripts submitted to the *Br J Ind Med* should conform to the uniform requirements for manuscripts submitted to biomedical journals (known as the Vancouver style).

The *Br J Ind Med*, together with many other international biomedical journals, has agreed to accept articles prepared in accordance with the Vancouver style. The style (described in full in *Br Med J*, 24 February 1979, p 532) is intended to standardise requirements for authors.

References should be numbered consecutively in the order in which they are first mentioned in the text by Arabic numerals above the line on each occasion the reference is cited (Manson¹ confirmed other reports²⁻⁵ . . .). In future references to papers submitted to the *Br J Ind Med* should include: the

names of all authors if there are six or less or, if there are more, the first three followed by *et al*; the title of journal articles or book chapters; the titles of journals abbreviated according to the style of *Index Medicus*; and the first and final page numbers of the article or chapter.

Examples of common forms of references are:

- 1 International Steering Committee of Medical Editors. Uniform requirements for manuscripts submitted to biomedical journals. *Br Med J* 1979;1:532-5.
- 2 Soter NA, Wasserman SI, Austen KF. Cold urticaria: release into the circulation of histamine and eosino-phil chemotactic factor of anaphylaxis during cold challenge. *N Engl J Med* 1976;294:687-90.
- 3 Weinstein L, Swartz MN. Pathogenic properties of invading micro-organisms. In: Sodeman WA Jr, Sodeman WA, eds. *Pathologic physiology: mechanisms of disease*. Philadelphia: W B Saunders, 1974:457-72.