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Note

Determination of two mercapturic acid metabolites of 1,3-dichloropropene in human urine with gas chromatography and sulphur-selective detection

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Z-1,3-Dichloropropene (*Z*-DCP) and *E*-1,3-dichloropropene (*E*-DCP) (Fig 1) are the active constituents of soil fumigants, such as Shell DD 95® and Telone II®. Biotransformation of *Z*- and *E*-DCP in animals has been shown to lead to the formation of N-acetyl-L-cysteine derivatives (mercapturic acids), which were excreted in the urine [^{14}C]-*Z*-DCP orally administered to rats was found to be excreted in urine for 75% as N-acetyl-S-(*Z*-3-chloropropenyl-2)-L-cysteine (*Z*-DCP-MA, Fig. 1) within 24 h [1]. Gas chromatography (GC) with different detection techniques (nitrogen-, sulphur- and mass-selective detection) has been shown to be applicable for the determination of *Z*- and *E*-DCP-MA in biotransformation studies in rats [2]. Intraperitoneal administration to rats of *Z*- and *E*-DCP in doses ranging from 25 to 450 μg/kg, resulted in urinary excretion of 55% of the dose as *Z*-DCP-MA and of 45% of the dose as N-acetyl-S-(*E*-3-chloropropenyl-2)-L-cysteine (*E*-DCP-MA, Fig 1) [2]

In the urine of five human volunteers, *Z*-DCP-MA has been measured after occupational exposure during application of Telone II® [3] However, this method has its limitations, since under the GC conditions applied, *Z*- and *E*-DCP-MA probably cochromatographed. Consequently, *Z*- and *E*-DCP-MA were probably determined together. In addition to that the internal standard, N-acetyl-S-(2-chloropropenyl-2)-L-cysteine, in principle could also be a bio-

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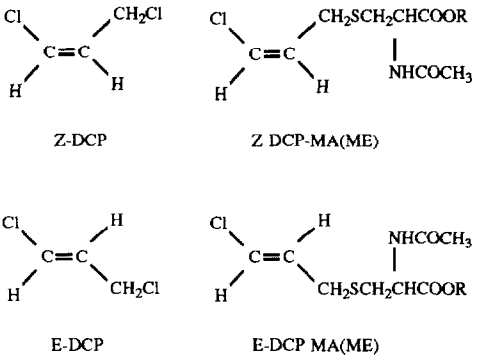

Fig 1 Structures of *Z*- and *E*-1,3-dichloropropene (*Z*- and *E*-DCP), the mercapturic acid metabolites N-acetyl-S-(*Z*- and *E*-3-chloropropenyl-2)-L-cysteine (*Z*- and *E*-DCP-MA, R=H) and the corresponding methyl esters (*Z*- and *E*-DCP-MAME, R=CH_3)

transformation product of 2,3-dichloro-1-propene, which may be present as an impurity in commercial DCP formulations [4]. An important reason to quantify urinary *Z*- and *E*-DCP-MA separately for biomonitoring purposes could be the supposed difference in direct mutagenicity of *Z*- and *E*-DCP in the *S typhimurium* assay [5]. To what extent other toxicological phenomena, e.g. carcinogenicity [6], are also stereoselective is still unclear, however

The major objective of this study was to develop a GC procedure to identify and quantify simultaneously the two mercapturic acids of *Z*- and *E*-DCP in human urine. The applicability of the method was evaluated in a field study of applicators occupationally exposed to soil fumigants containing *Z*- and *E*-DCP in The Netherlands.

EXPERIMENTAL

Materials

Z-DCP (97%), *E*-DCP (97%) and N-acetyl-L-cysteine (98%) were purchased from Janssen Chimica (Beerse, Belgium) Creatinine stock solutions were obtained from Sigma (St. Louis, MO, U S.A.). Synthetic urine sample jars were obtained from Thovadec (Nieuwkoop, The Netherlands).

Synthesis and purity of reference compounds

The purity of the commercially obtained *Z*- and *E*-DCP was checked by gas chromatography–mass spectrometry (GC–MS) *Z*-DCP contained some *E*-DCP and vice versa. *Z*-DCP also contained 1,2-dichloropropane and a minor unidentified component. *E*-DCP contained the same unidentified component as *Z*-DCP.

The syntheses of *Z*- and *E*-DCP-MA were carried out by treating N-acetyl-L-cysteine with *Z*- or *E*-DCP in sodium methanolate [2] The free carboxylic

acid contents, determined with an acid–base titration using phenol red as indicator, were 92 ± 0.8 and 96 ± 1.5% (mean ± S.D., $n = 3$) for *Z*- and *E*-DCP-MA, respectively. By adding ethereal diazomethane to solutions of *Z*- or *E*-DCP-MA in methanol, the methyl esters *Z*- and *E*-DCP-MAME (Fig 1) were prepared ^{1}H NMR and electron-impact mass spectra of both *Z*- and *E*-DCP-MA and their methyl esters corresponded to reference data previously published [2]

The internal standard N-acetyl-S-(benzyl)-L-cysteine (BEMA) was synthesized according to Van Bladeren et al. [7]. Ethereal diazomethane was generated from N-methyl-N-nitrosoureum and 40% potassium hydroxide in diethyl ether [8].

Wall-coated open tubular (WCOT) column gas chromatography

GC with sulphur-selective detection (GC–FPD) was performed on a Packard 428 gas chromatograph, equipped with a Packard Model 906 flame-photometric detector (S-mode), a ball-valve solid injector and a fused-silica WCOT CP Sil 19CB column (25 m × 0.25 mm I.D., d_f 0.18 μm) (Chrompack, Middelburg, The Netherlands). The gas chromatograph was connected to a Hewlett-Packard 3390A integrator. The temperatures of the injection port, column and detector were 290, 210 and 250°C, respectively. The helium carrier gas flow-rate was ca. 3 ml/min In the detector the hydrogen flow-rate was 142 ml/min and the air flow-rates were 55 and 165 ml/min.

To confirm the identity of the eluting GC peaks, a Finnigan 9610 gas chromatograph coupled with a Finnigan 4000 mass spectrometer operated with a Finnigan Matt NOVA/4 computer system was used. Two columns connected with each other were used. The first (35 m × 0 30 mm I.D., d_f 0.36 μm) was a glass capillary column coated with OV-1701, the second was a fused-silica column (15 m × 0.32 mm I.D., d_f 0.36 μm) coated with SE-30 [9]. The injection port and transfer-line temperatures were both 250°C. When checking the purity of *Z*- and *E*-DCP the column temperature was 60°C. During the analysis of isolated and derivatized *Z*- and *E*-DCP-MA from human urine the column was temperature-programmed, the initial temperature being 100°C (1 min), the programming rate 20°C/min and the final temperature 240°C (15 min). Helium was used as carrier gas. For the identification of the metabolites, electron-impact ionization was applied (electron energy 70 eV, emission current −0.20 mA).

Collection of human urine

Twelve applicators collected urine samples before, during and for 24 h after finishing soil fumigation with DCP. The urine samples were stored in a cold (≤4°C) and dark place on the same day. Laboratory transport was within two days after collection and the storage temperature afterwards was −18°C. Creatinine in urine was determined according to Tietz [10]

Isolation and derivatization of Z- and E-DCP-MA

To 5 ml of human urine, 6.9 or 69.5 μg of the internal standard BEMA were added, depending on the expected *Z*- and *E*-DCP-MA concentrations. The urine samples were acidified with 350 μl of 2 *M* hydrochloric acid, resulting in a pH 1–2. Two 2-ml volumes of ethyl acetate were used for extraction by vortex-mixing for 30 s. Thereafter centrifugation took place at 2500 *g* for 5 min. After subsequent evaporation of the combined ethyl acetate layers under a gentle nitrogen flow in a water-bath at 37°C, the residues were dissolved in 500 μl of methanol. The residues were methylated by adding 1 ml of an ethereal diazomethane solution, which was allowed to react for 1 h at room temperature. After evaporation of the ethereal diazomethane solution the residues were finally redissolved in 200 or 500 μl of ethyl acetate, for low or high concentrations of mercapturic acid, respectively.

Quantitative determination of Z- and E-DCP-MA

Internal standard calibration curves were prepared by dissolving *Z*- and *E*-DCP-MA in concentrations ranging from 0.1 to 2 μg/ml or ranging from 1 to 60 μg/ml of human urine. To urine samples containing low concentrations of *Z*- and *E*-DCP-MA (i.e ≤ 2 μg/ml), 6.9 μg of the internal standard BEMA were added, whereas to urine samples containing high concentrations of *Z*- and *E*-DCP-MA (i e. ≥ 1 μg/ml), 69 5 μg of BEMA were added. Isolation and derivatization procedures were executed as described above.

GC–FPD peak-area ratios of methylated *Z*- and *E*-DCP-MA and the methylated internal standard BEMA were plotted double-logarithmically in order to obtain straight calibration curves [11]. Extraction recoveries of *Z*- and *E*-DCP-MA were calculated from the ratio of the slopes of standard calibration curves in ethyl acetate and external standard calibration curve (in which BEMA-methyl ester was added as external standard to the residues dissolved in ethyl acetate).

Stability of Z- and E-DCP-MA in urine

A stability study of *Z*- and *E*-DCP-MA was performed by storing spiked human urine (1–15 μg/ml urine) in urine sample jars at different temperatures (−18°C, 0°C and room temperature), in dark and daylight and for different time intervals (0, 24, 48 h and one year).

RESULTS AND DISCUSSION

The major objective of this study was to develop a GC procedure to analyse simultaneously *Z*- and *E*-DCP-MA in human urine and to evaluate its applicability to urine samples of applicators occupationally exposed to *Z*- and *E*-DCP containing soil fumigants.

Stability and quantitative determination of Z- and E-DCP-MA in human urine

Z-DCP-MA and E-DCP-MA proved to be stable in human urine provided that samples were stored in a cold (−18°C) and dark place. Prolonged storage at room temperature and in light negatively influenced the recovery and the linearity of the calibration curves of both Z- and E-DCP-MA. Recoveries of Z- and E-DCP-MA from human urine samples after one year dark storage at −18°C were 105±4% (mean±S D., $n=5$) and 105±3% (mean±S.D., $n=5$), respectively.

Fig 2A–C show typical GC–FPD traces of Z- and E-DCP-MA in blank human urine and urine spiked with a low or a high concentration of Z- and E-DCP-MA. Fig. 2D shows a GC–FPD profile of a typical sample from a DCP applicator

The detection limits of Z- and E-DCP-MA in human urine following the extraction and derivatization procedure described above were determined by injecting 5 μl of the ultimate ethyl acetate residues of 200 μl The lowest detectable concentrations in human urine were found to be 107 ng/ml for Z-DCP-MA (coefficient of variation, C.V.=6.9%, $n=8$) and 115 ng/ml for E-DCP-MA (C.V =7.5%, $n=8$) The absolute amounts injected on the column in these cases were 9 and 10 ng for Z- and E-DCP-MA, respectively In principle, lower concentrations of both mercapturic acids in urine could be detected, although the C.V values then exceeded 10%. The detection limits reached in this study compare favourably with those for the same mercapturic acids previously obtained with GC–FPD in rat urine, namely 55 and 20 ng/ ml for Z- and E-DCP-MA, respectively [2] Other published analytical techniques and detection limits for mercapturic acids in human urine include high-performance liquid chromatography with UV detection of N-acetyl-S-(phenyl)-L-cysteine up to 1500 ng/ml [12], GC with nitrogen-selective detection of N-acetyl-S-(N-methylcarbamoyl)-L-cysteine up to 200 ng/ml [13] or GC–MS with selected-ion monitoring of BEMA (m/z 176 and 208) or N-acetyl-S-(*o*-methylbenzyl)-L-cysteine (m/z 176 and 222) up to 500 and 300 ng/ml, respectively [14].

Double-logarithmically plotted internal standard calibration curves for concentrations ranging from 1 to 60 μg/ml Z- and E-DCP-MA in human urine showed good linear relationships, $r \geq 0.9899$ and 0 9913, as well as low inter-day C V values of 6 and 4% ($n=4$), respectively. Whereas linearity was also good in the concentration range 0.1–2 μg/ml Z- and E-DCP-MA in human urine, $r \geq 0$ 9983 and 0.9975, the inter-day C V values were 8 and 16% ($n=4$) The GC–FPD response of Z-DCP-MA was found to be identical with that of E-DCP-MA. Extraction recoveries of Z- and E-DCP-MA from spiked human urine were found to be 69±5 and 70±6% (mean±S D , $n=4$), respectively

Identification and quantitative determination of Z- and E-DCP-MA in the urine of applicators

In order to identify Z- and E-DCP-MA, GC–MS was used. Fig 3 shows mass chromatograms of the ions at m/z 117 and 176, and the reconstructed ion cur-

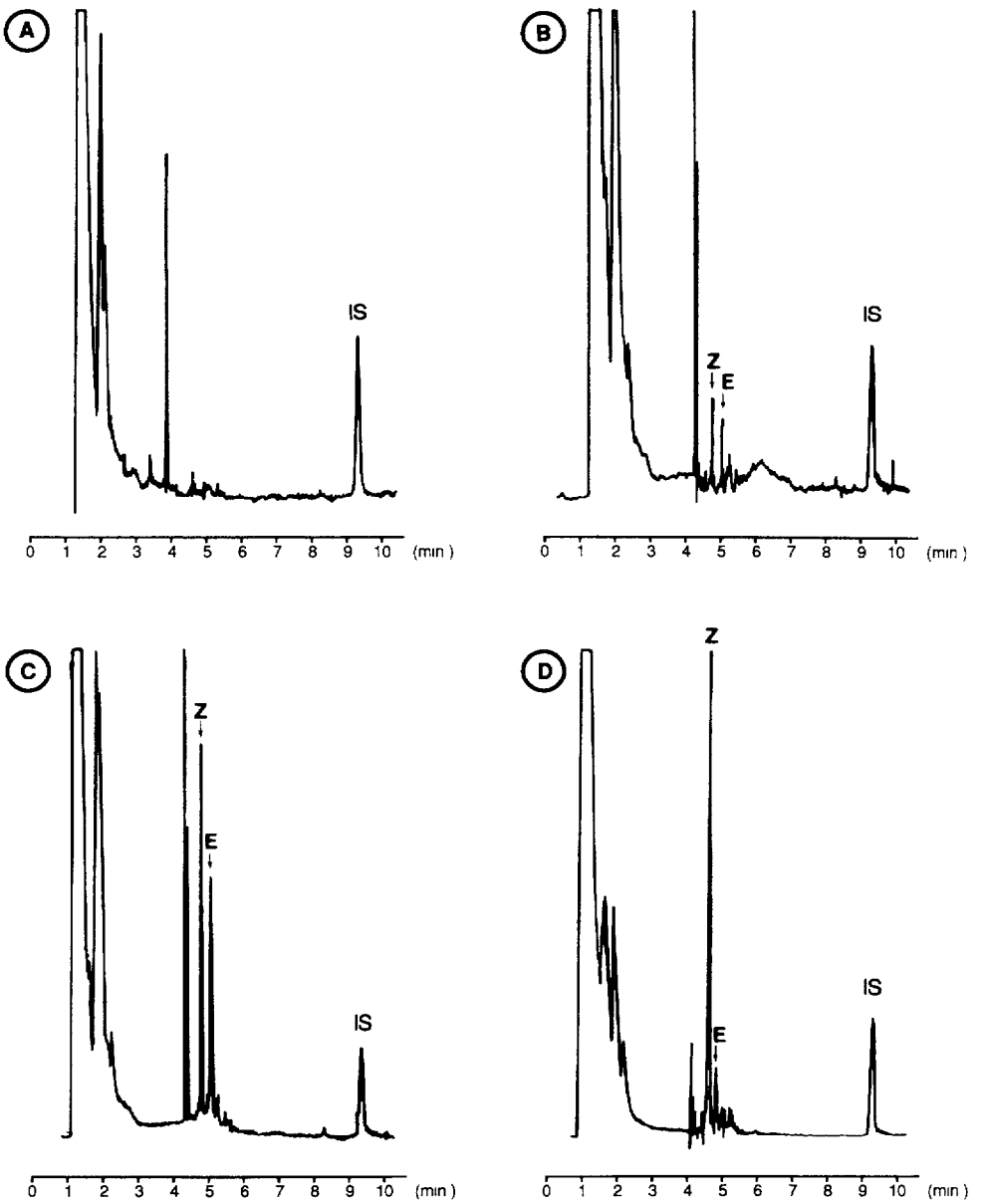

Fig 2 GC–FPD profiles of (A) a blank human urine, (B) human urine spiked with 107 and 115 ng/ml *Z*- and *E*-DCP-MA and (C) 534 and 574 ng/ml *Z*- and *E*-DCP-MA, respectively, and (D) a urine sample from an applicator exposed to DCP In the latter chromatogram the peaks of *Z*- and *E*-DCP-MA correspond to 1020 and 260 ng/ml, respectively Peaks Z=*Z*-DCP-MA, E=*E*-DCP-MA, IS=the internal standard BEMA The column and the chromatographic conditions are described in Experimental

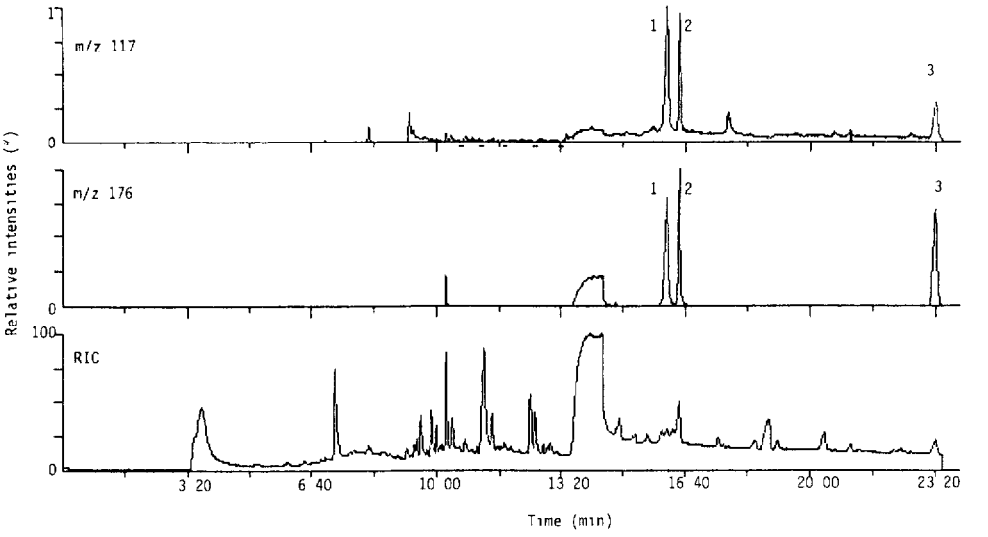

Fig 3 GC–MS single-ion traces of *m/z* 117 and *m/z* 176 and the reconstructed ion current (RIC) chromatogram of a urine sample from an applicator Peaks 1 = *Z*-DCP-MA, 2 = *E*-DCP-MA, 3 = the internal standard, BEMA The column and chromatographic conditions are described in Experimental

rent (RIC) chromatogram of a representative urine sample of an applicator In the RIC chromatogram *Z*- and *E*-DCP-MAME are hardly visible owing interfering peaks from endogenous urinary components. Nevertheless, with suitable background subtraction, complete electron-impact mass spectra of *Z*- and *E*-DCP-MAME could be constructed from the total ion current Comparison of the electron-impact mass spectra and the retention times with those of synthetic *Z*- and *E*-DCP-MA and standard addition of synthetic *Z*- and *E*-DCP-MA confirmed the identity of *Z*- and *E*-DCP-MA as metabolites of *Z*- and *E*-DCP in human urine Using GC–MS with selected-ion monitoring of four ions previously characterized as typical key ions for mercapturic acids, i e *m/z* 88, 117, 144 and 176 [2,15], the presence of *Z*- and *E*-DCP-MAME was further confirmed.

In order to evaluate the practical applicability of the method, several urine samples of applicators were analysed In Fig. 4 the time courses of *Z*- and *E*-DCP-MA concentrations during two days, corrected for creatinine concentration, are depicted for one applicator On the first day of urine collection, neither *Z*- nor *E*-DCP-MA was detectable At that time the applicator had not used any soil fumigants for five days. On two consecutive days, *Z*- and *E*-DCP-MA concentrations in urine rose during field application of DCP and decreased during the day after exposure The observed difference in excretion between *Z*- and *E*-DCP-MA could be due to a difference in exposure, uptake, metabo-

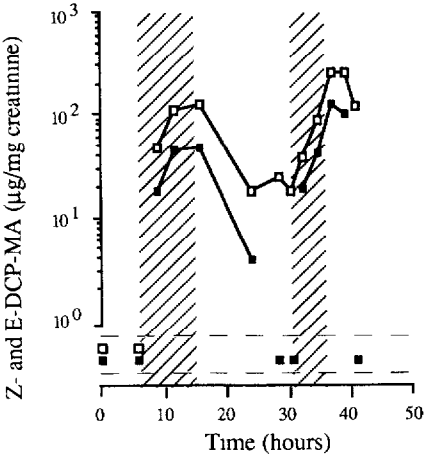

Fig 4 Time courses of *Z*-DCP-MA (□) and *E*-DCP-MA (■) concentrations in urinary spot-samples, corrected for creatinine content, during two consecutive days of an applicator applying soil fumigants containing *Z*- and *E*-DCP Urine samples in which no *Z*- and *E*-DCP-MA could be detected are indicated between dashed lines The shading indicates the exposure periods

lism or excretion of both isomers. The analytical method proved to be sufficiently sensitive and selective for the measurement of two mercapturic acid metabolites of *Z*- and *E*-DCP in human urine

CONCLUSION

Capillary GC, in combination with mass-selective and sulphur-selective detection, was found to be suitable for the qualitative and quantitative analysis of *Z*- and *E*-DCP-MA in the urine of applicators occupationally exposed to *Z*- and *E*-DCP Measurements of these two urinary metabolites, therefore, might be used for exposure assessment if the relation between uptake of *Z*- and *E*-DCP and urinary *Z*- and *E*-DCP-MA excretion in humans has been elucidated.

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