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RAPID COMMUNICATIONS

IDENTIFICATION OF S-1,2-DICHLOROVINYL-N-ACETYL-CYSTEINE AS A URINARY METABOLITE OF TRICHLOROETHYLENE: A POSSIBLE EXPLANATION FOR ITS NEPHROCARCINOGENICITY IN MALE RATS

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Introduction

The carcinogenicity of trichloroethylene (Tri) has been extensively debated over the past ten years. In recent long-term gavage studies, very high doses of Tri increased the rate of hepatocellular carcinoma in $B6C3F_1$ mice and caused renal tubular adenocarcinoma in male Fischer 344 rats, a very rare spontaneous tumor (1). The molecular mechanisms responsible for the different susceptibility of mice and rats have not yet been satisfactorily eludicated. Tri has been shown to be metabolised by liver cytochrome P-450 with the formation of short lived reactive intermediates in both rats and mice (2). The observed liver carcinogenicity in mice has been attributed to the higher rate of biotransformation in this species (3). In contrast, reactive intermediates formed by cytochrome P-450 mediated oxidations cannot explain the nephrocarcinogenicity of Tri in rats, because P-450 activity in the kidney is much lower than in the liver (4). An alternative mechanism has been proposed to explain the organ-specific toxicity of several halogenated olefins: direct conjugation of the olefin with glutathione (GSH) and further processing to haloalk(en)yl-cysteines and cleavage of the C-S bond by enzymes concentrated in the renal tubule with the formation of reactive intermediates (5). No data on conjugation of Tri with GSH in mammals are available in the literature, although the nephrotoxicity of dichlorovinyl-cysteine (DCVC) which is expected to be formed from the putative GSH-conjugate via the mercapturic acid pathway has been intensively investigated (6).
GSH-conjugation of Tri should result in the excretion of the mercapturic acid N-acetyl-DCVC in the urine, which would provide a hint as to the mechanism of nephrocarcinogenicity of Tri in male rats. We have therefore reinvestigated the biotransformation of Tri giving special attention to the occurence of N-acetyl-DCVC in male rats.

Materials and Methods

1,2-^{14}C-trichloroethylene was purchased from New England Nuclear (Dreieich, FRG). The sample delivered contained several radioactive impurities and was purified as described elsewhere (2). S-1,2-dichlorovinyl-N-acetyl-cysteine was synthesized by acetylation of dichlorovinyl-cysteine (prepared as described in 7) with acetic acid anhydride/pyridine. The identity and purity of the reference compound was confirmed by 1-H-NMR and GC/MS (see Fig. 1). 1-H-NMR (CD_3CN, int. TMS): δ (ppm) 1.95 3 H; 3.3, 2 H; 4.7, 1 H; 6.6, 1 H; 6.9, 1 H. HPLC-grade solvents were used for all HPLC-separations.

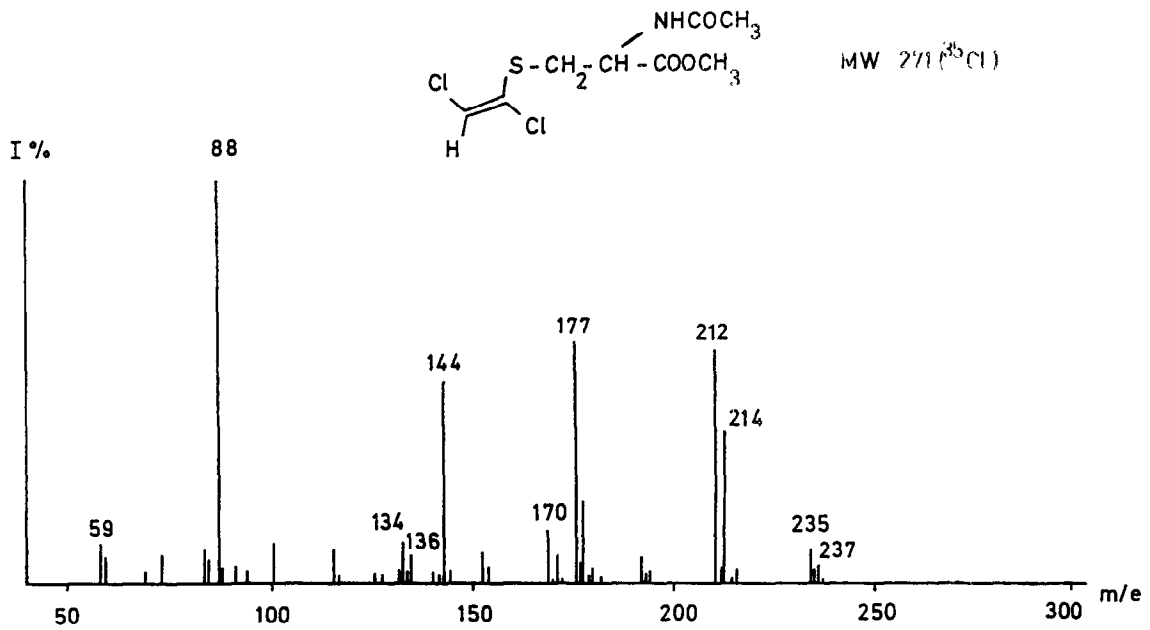

Figure 1: Electron-impact mass spectrum of synthetic dichlorovinyl-N-acetyl-cysteine methyl ester

Animals and treatment: Male Sprague-Dawley rats (300-360 g) fasted for 24 hours (Institut fuer Versuchstierkunde, Hannover, FRG) were administered single doses of ^{14}C-Tri (400 mg/kg) in corn oil by stomach tube between 9 and 10 a.m. and immediately transferred to an all-glass metabolic cage. Dry air was drawn through the cage at a constant rate (400 ml/min). Standart diet (Altromin^{R}) and water were supplied ad libitum.

Sampling of urine and measurement of radioactivity: Urine was collected for 72 hours and kept at 4°C during the sampling period. Aliquots were mixed with liquid scintillation cocktail (Quickszint 212, Zinsser, Frankfurt, FRG) and counted in a liquid scintillation counter (Tricarb 4000, Packard Instruments, Downers Grove, USA).

Isolation of urinary fractions expected to contain N-acetyl-DCVC: Two volumes (v/v) of ethanol were added to the collected urine and stored at 4°C for 24 hours. The precipitated protein was discarded and the supernatant concentrated at reduced pressure, followed by ether extraction at pH = 2. The extracts were concentrated and subjected to further fractionation by HPLC (Waters, Milford, USA) under UV control at 235 nm. Purification of fractions (0.5 ml each) expected to contain N-acetyl-DCVC was performed in three consecutive steps: Preparative separations (A+B) were performed using steel columns (25 x 0.8 cm) filled with Partisil ODS - III (Whatman, Maidstone, England):
A: initial conditions water, 5% methanol, 0.1% trifluoroacetic acid (TFA); final conditions 100% methanol; linear gradient 40', flow rate 3 ml/min.
B with fractions 45 - 60: 40 % methanol - 80 % methanol in water (0.1% TFA), linear gradient 30', 3 ml/min.
For purification step C (with fractions 24 - 27 from B), a Waters RCM-module with a Nova-Pak C-18 cartridge (5 mm I.D.) was used.
C: 20 % acetonitrile - 60 % acetonitrile in water (0.1% TFA), linear gradient, 20', 1.4 ml/min.

Gaschromatography/mass spectrometry: The dry residues (evaporation of the solvent at reduced pressure) of fraction 5 and 6 from step C were treated with BCl_3/methanol for transformation of N-acetyl-DCVC to the volatile methyl ester. GC/MS was performed on a Finnigan (San Jose, USA) 4510 GC/MS system. A DB-5 (0.1 µm film) coated fused silica capillary column (25m x 0,25mm, J & W Scientific, Rancho Cordova, USA) was used for gas chromatographic separations (splitless injection;

linear temperature programme 10°/min, initial temperature 50°C). Retention time of the synthetic N-acetyl-DCVC methyl ester was 580 sec. under these conditions. Mass spectrometric data were processed by a Finnigan Incos MAT data system. Electron-impact mass spectra were recorded at 70 eV.

Results and Discussion

The radioactivity profiles obtained from the first HPLC-separation of the collected 72 h urine indicated a small peak (less than 0.1 % of total radioactivity injected) identical to the retention of the synthetic reference N-acetyl-DCVC (data not presented). The radioactive compound in this peak was subjected to the three step HPLC-purification procedure. The fractions 5 and 6 obtained from step C were collected and pooled. After evaporation of the solvent, the dry residue was esterified and subjected to GC/MS analysis. The very low concentrations of the metabolite did not allow for obtaining a mass spectrum with all the expected fragments. We therefore monitored the four most intensive and representative fragments (m/e = 144, 177, 212, 214) present in the mass spectrum of the synthetic reference (see Fig. 1) for multiple ion detection during the gas chromatographic separation of the sample. Fig. 2 shows the relative intensities of the monitored fragments recorded during separation of the sample.

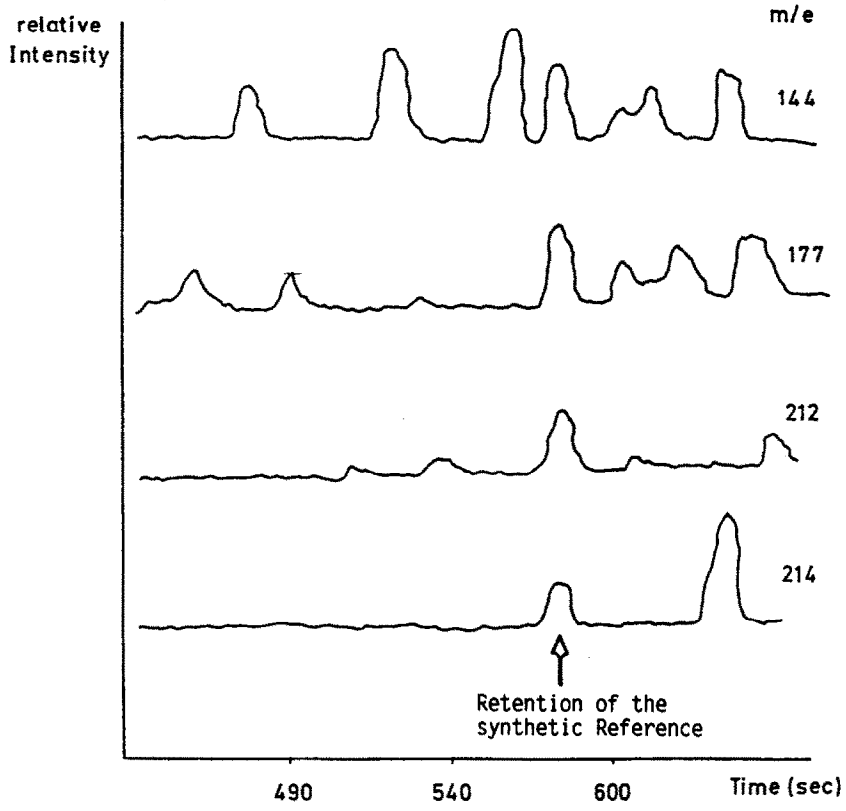

Figure 2: Monitoring of selected fragments of N-acetyl-DCVC methyl ester during gaschromatographic separation of a purified urine sample from rats receiving 400 mg/kg trichloroethylene

The mass fragments recorded (retention time 572-585 sec.) were identical to those of the synthetic reference with respect to typical fragments and their relative intensities. In addition, the retention time of the sample and the synthetic reference were identical under the gas chromatographic conditions used.

The major metabolites of Tri present in the urine (1,1,1-trichloroethanol, trichloroacetic acid, oxalic acid, N-hydroxyacetyl-aminoethanol) were characterised by their retention characteristics in the radioactivity profiles of HPLC-separations and were found to be identical to those obtained in

previously described experiments (2). Further minor fractions have been observed but not yet been identified.

The reference substance of N-Ac-DCVC is most probably the trans-isomer but a definite discrimination is not yet possible. The unequivocal identification of N-acetyl-DCVC after high doses of Tri in rats indicates, besides the well known P-450 catalysed oxidative metabolism, the existence of a novel pathway of biotransformation of that compound which has been overlooked in the past. It must be regarded as an activation pathway, since DCVC, the precursor of N-acetyl-DCVC exerts acute nephrotoxic as well as genotoxic effects (8). It might therefore account for the nephrocarcinogenic effect of Tri as demonstrated by others (1), at least for the present conditions of exposure : high doses by gavage to male rats, conditions which were used in the carcinogenicity bioassays as well as in our metabolism studies.

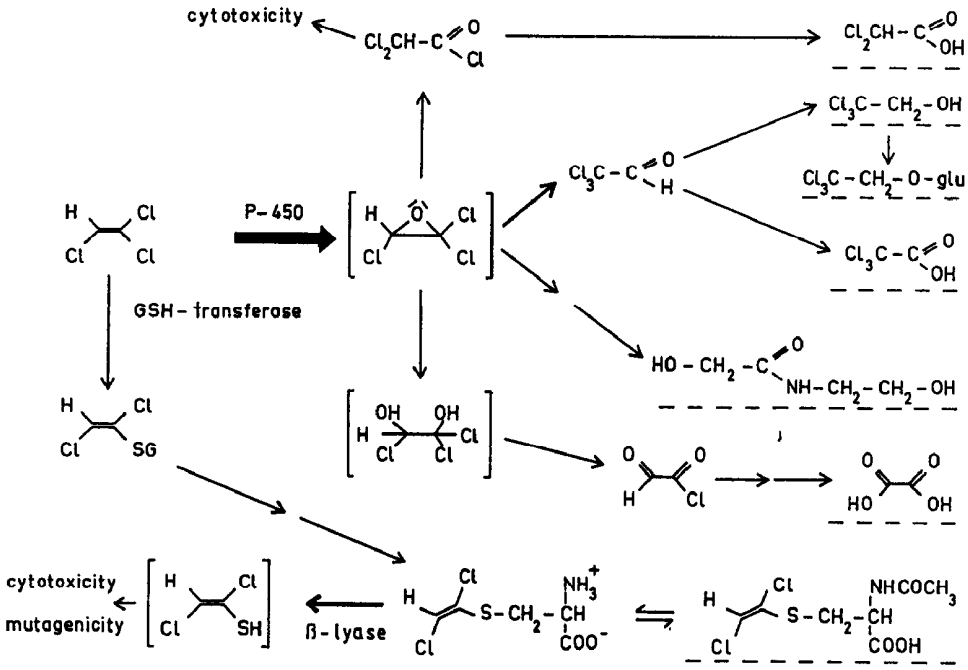

Figure 3: Metabolic pathways of trichloroethylene

The first step in DCVC formation (Fig. 3), conjugation with glutathione, most probably takes place in the liver. The GSH-conjugate is further processed in the epithelium of the renal tubules (9) where the enzyme which catalyses the final step, ß-lyase, is also localised, thus explaining the high organ specificity. The same sequence of events has also been postulated for the nephrotoxic and nephrocarcinogenic compound hexachlorobutadiene (10).

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