

**PILOT STUDY FOR BIOLOGICAL MONITORING  
OF 1,3-DICHLOROPROPENE**

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## Chapter 17

### Pilot Study for Biological Monitoring of 1,3-Dichloropropene

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A pilot study was performed to estimate excretion patterns of the metabolite of dichloropropene (DCP), N-acetyl-S-(cis-3-chloroprop-2-enyl)-cysteine (3CNAC). Urine from 3 applicators was collected every 2-16 hours for 3 days, while personnel air concentrations of DCP were determined by EC-GC. 3CNAC in urine was extracted, derivatized and measured on capillary gas chromatograph-mass spectrometry by monitoring the two abundant ions 117 and 176 m/e. Sensitivity was 0.1 ug/mL of urine. Air concentrations of DCP ranged from 0-10.3 ppm. Urine concentrations of 3CNAC ranged 0.9-17.1 ug/mL. Excretion of peak concentrations of metabolite followed peak exposure by variable periods of time (0-16 hours). Cumulative daily excretion of 3CNAC increased in proportion to cumulative daily air exposure.

1,3-Dichloropropene (DCP) is an agricultural fumigant applied by pressure injection into soil for pre-implantation eradication of nematodes. Commercial preparations contain 98% of approximately equal parts of cis and trans DCP. Because of its vapor pressure (28 mmHg), the primary route of exposure to human workers is suspected to be pulmonary. Low ppm air concentrations have been documented during loading, mixing and applications processes (1,2). Both isomers are metabolized to their respective mercapturic acids in rats (3-5). We have demonstrated previously that monitoring of human applicators is possible by measuring the metabolite N-acetyl-S-(cis-3-chloroprop-2-enyl)-cysteine (3CNAC) (2). Protection and biological monitoring of workers is of concern since DCP has produced forestomach squamous and urinary bladder transitional cell cancers following chronic oral gavage studies in rats and mice (6). Subchronic inhalational studies in rats and mice at

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90 or 150 ppm have demonstrated hyperplasia respiratory epithelium, degeneration of nasal mucosa and hyperplasia of transitional cell epithelium in the urinary bladder (W.T. Stott, Dow Chemical Co., personal communication). Chronic inhalation studies are not published, but increased respiratory neoplasia are suggested (W.T. Stott, personal communication). DCP is an indirect mutagen (7-11) which binds (8,9) and depletes sulfhydryl groups in the liver and kidney (12). Earlier studies indicated that the rat kidney may show toxic effects in the renal tubular epithelium following inhaled concentrations as low as 3 ppm after chronic inhalation (13). This effect has not been confirmed in two subsequent subchronic animal studies following inhalation of concentrations as high as 150 ppm (14,15). However, a kinetic study by one of these groups has shown that a 3 hour exposure to 90 ppm reduces kidney sulfhydryl content 31% (12). While differences in studies may be attributed to differences in exposure duration or species differences, histologic and standard clinical chemistry assays used in chronic studies may not have the sensitivity to demonstrate such subtle renal effects.

Safe air concentrations of DCP which protect workers are not known. Estimates of possible carcinogenic risk based on possible air concentrations and animal studies do not yield real or specific thresholds. Twenty-four hour cumulative urinary excretion of 3CNAC increases in rough proportion to increasing air concentrations \* time products (2). Single random determinations of urinary 3CNAC only indicate that some systemic exposure has occurred, but are not indicative of known exposure thresholds or toxic effects. It is also not known how quickly excretion of 3CNAC follows pulmonary exposure.

Knowledge of timing and toxic effects would aid in determining appropriate urine collection procedures. As a pilot to a larger study, we evaluated three workers by measuring personal air concentrations of DCP urinary excretion of 3CNAC and of N-acetyl-glucosaminidase (NAG). The latter is an enzyme released from injured tubular epithelium in the kidney. It has been studied as a more sensitive indicator of renal injury following a wide variety of nephrotoxins (16-18). Analytical concerns in measuring 3CNAC will also be discussed.

#### Materials and Methods

Protocol. Three male applicators of DCP were studied for 2-3 days. Field collections were done under the auspices of the Worker Health and Safety Branch of California Department of Food and Agriculture. Application rates of DCP were 12 gallons per acre. The soil was sandy and dry with air temperatures exceeding 100°F for most of the day. The work periods of potential exposure to DCP varied from 3.5 - 10 hours/day. Workers wore light cotton clothes during application of DCP. During loading, chemical resistant gloves, apron, boots, and NIOSH-approved respirators were worn. Dermal exposure from

small spills during these closed loading operations may have occurred, but none were recorded. The extent of the contribution of dermal exposure to urinary 3CNAC excretion is unknown.

Applicators were chosen only on a first available basis in the locale where DCP was being applied. No attempt was made to exclude a subject with prior exposure to DCP in this pilot study. At the field site, prior to beginning work, the applicator was attached with a personal air sampling device and instructed as to collection of urinary specimens. Operator breathing zone air samples were drawn by personal air sampling pumps (MSA Fixt Flo) through charcoal adsorbent tubes (SKC #226-09, 400/200 mg) via tygon tubing. Tube openings were positioned down across the chest and did not interfere with work habits. Pumps were calibrated to 1 L/min using a Kurz 540S mass flow calibrator. Sorbent tubes were changed at up to four hours or when there was an obvious change in work practice (loading vs. repair) intervals. These tubes were capped and stored on dry ice, then refrigerated ( $-20^{\circ}$ ) until analysis. Procedures, flow rates and breakthrough volumes are given elsewhere (19,20).

Urines were collected by instructing the applicators to urinate into a separate 500 mL opaque polyethylene container for each voiding. Each container was frozen on dry ice and then  $-70^{\circ}\text{C}$  until analysis. Urine collection occurred only when the applicator would empty his bladder. Thus, collection intervals were variable and spanned 2-16 hours. Urine collection periods did not coincide with intervals for air collections.

Analysis of air concentrations of DCP. Amounts of DCP in sorbent tubes were determined in accord with established procedures (1,19,20) that includes elution of tubes with carbon disulfide mixed with an internal standard and direct injection onto electron capture gas chromatograph. Sensitivity and precision of these determinations is 0.1 ug collection (column).

Urinary NAG Determinations. Urinary activities of NAG were determined by the manual method of Piagen et al (1978) with modifications incorporated by Al-Bander et al (1986). This is a fluorometric method which measures the hydrolysis of 4-methylumbelliferyl-Beta-D-glucosaminide. Calculated activity in nanomoles/hr/mL of urine is expressed in terms of milligrams of creatinine excreted (nanomoles/hr/mg), since this corrects for variability due to urine flow rates (23). Creatinine concentrations were determined by the Jaffe reaction (24). Intraassay coefficients of variation were <5% at 50 nmol/hour/mg of creatinine. The normal reference interval is <100 nmol/hour/mg.

Urinary 3CNAC Determinations. Synthesis of the major metabolite, N-acetyl-S-cis-3-chloroprop-2-enyl)-cysteine (3CNAC), and the isomeric internal standard, N-acetyl-S-2-chloroprop-2-enyl)-cysteine (2CNAC), from N-acetyl cysteine and the appropriate dichloropropene has been described elsewhere (2,4). Purification procedures included multiple recrystallizations with verification of the product and its purity by thin layer chromatography, flame ionization gas chromatography, electron capture gas chromatography (EC-GC), melting points, infrared spectra, nuclear magnetic resonance spectra and GC-MS. Analytic grade solvents were purchased from either Mallinkrodt, Burdick and Jackson or Aldrich. Stock standards of the 3CNAC and 2CNAC were prepared in methanol or acetone at concentrations of 1.0 mg/ml for use in chromatography and spiking blank urine solutions as standards.

Prior to developing a capillary GC-MS method for quantitation, other gas chromatographic techniques were employed, including packed column chromatography with flame ionization, nitrogen phosphorus and mass selective detectors; and also direct injection into various capillary columns with electron capture detection. Most of these other chromatographic techniques were unsuitable for analysis of urines containing 3CNAC due to endogenous interferences or non-symmetrical peak shapes. On-column injection into wide bore (0.5 mm) columns of methyl silicone or methyl phenyl silicone were generally unsuitable with EC-GC instrumentation due to poor peak symmetry and sensitivity. However, with split injection into narrow bore methyl silicone columns, acceptable sensitivity (0.5 ug/mL) has been achieved, but endogenous unknown urinary constituents limit the utility of this method.

Previously, extraction of 3CNAC from acidified urine relied on a diethyl ether extraction (2). To improve cleanup and yield, different extraction techniques were applied. Urine or water (5.0 mL) containing 100 ug/mL of 3CNAC were acidified to pH 1 with addition of 0.6 mL 6N HCl. Ten mL of each solvent was added, gently shaken for 5 minutes and centrifuged at 2000 g for 10 minutes. Eight mL of solvent phase was evaporated at 45°C under nitrogen. Following diazomethane derivatization and quantitation on EC-GC, peak areas were compared to 3CNAC standards. Blank unspiked urines were carried through to estimate interferences at the retention time of 3CNAC. Each solvent gave the following results (% recovery, % urinary interference): dichloromethane (16%, 15%), diethyl ether (10%, 100%), chloroform (30%, unknown), t-butyl methyl ether (80%, <10%), ethylacetate with saturated NaCl in aqueous phase (<10%, unknown). Ion pair extractions (tetramethylammonium salts in various solvents) and ion exchange column separation (bonded quaternary ammonium) gave poor recoveries of the 3CNAC carboxylic anion. Many different derivatization techniques following initial extraction were investigated. However, only dimethylformamide dipropyl acetal (Propyl-8, Pierce Chemical Co.), or diazomethane (generated from Diazald, Aldrich Chemical

Company) yielded 100% propyl and methyl ester derivatives, respectively. Following extraction and derivatization, an extraction-wash was employed to further clean up the sample. Chlorobutane partitioned with water gave better results than hexane, toluene or more polar solvents.

Procedure. Frozen urine is thawed to room temperature and 1.0 ml is mixed with 0.5 ml of 6 N HCl and 10  $\mu$ L of the stock internal standard (2CNAC, 1 mg/ml) in a 13 x 100 mm screw top borosilicate tube. Standards are prepared by spiking urine with appropriate quantities of the stock 3CNAC methanolic solution. Standard concentrations were 0, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0  $\mu$ g/mL of urine. Two ml of t-butyl methyl ether (TBME) is added to each tube, capped and extracted for 10 minutes while shaking. All samples are centrifuged for 10 minutes at 2000 g. The TBME phase (1.5 mL) is transferred to a new labeled tube and evaporated under nitrogen at 40°C. In an ice bath, 200  $\mu$ L of an ether solution of diazomethane is added at 5°C and capped. Following a reaction time of 30 minutes, the ether and excess diazomethane are evaporated to dryness under nitrogen flow at room temperature. Two ml of n-butyl chloride is added, mixed, followed by 1 ml of water. This extraction/wash is shaken for 5 minutes and then centrifuged for 10 minutes at 2000 g. The n-butyl chloride (1.5 mL) is evaporated under nitrogen at room temperature. Toluene (300-600  $\mu$ L) is added to the residue for GC-MS analysis.

Capillary GC-MS instrumentation included a Hewlett-Packard 5970B quadrupole mass selective detector connected to a 5890 gas chromatograph. The fused silica column was 12 m x 0.2 mm and coated with cross-linked methylsilicone. Helium carrier gas flow was 1 mL/min. The injector was heated to 200°C and operated in the splitless mode. The column was heated at 70°C for 4 minutes, then increased at 20°C/min to 225°C for a total program time of 12 minutes. The quadrupole was calibrated with perfluorotributylamine at masses of 69, 219, and 502. The electron multiplier voltage was 1600 volts and ionization voltage was 70 keV. The quadrupole was run in a low resolution mode with mass peak widths at half height (resolution) were 0.5 AMU. The ions monitored were 117 and 176 with a dwell time of 50 msec. One  $\mu$ L of the toluene sample was injected by a Hewlett-Packard 7673 automatic sampler. After standards calibration, all samples are determined in the same run. Controls were run every 8-10 samples. Quantitation was performed by comparing peak height ratios of 3CNAC methyl ester/2CNAC methyl ester for the ion monitored (either 117 or 176) to a standard curve of peak height ratios for the standards.

### Results

Urinary 3CNAC Procedure. Figure 1 shows electron capture gas chromatogram of a blank urine containing no added 3CNAC and a urine of a subject containing 10  $\mu$ g/mL of 2CNAC and 20  $\mu$ g/mL of

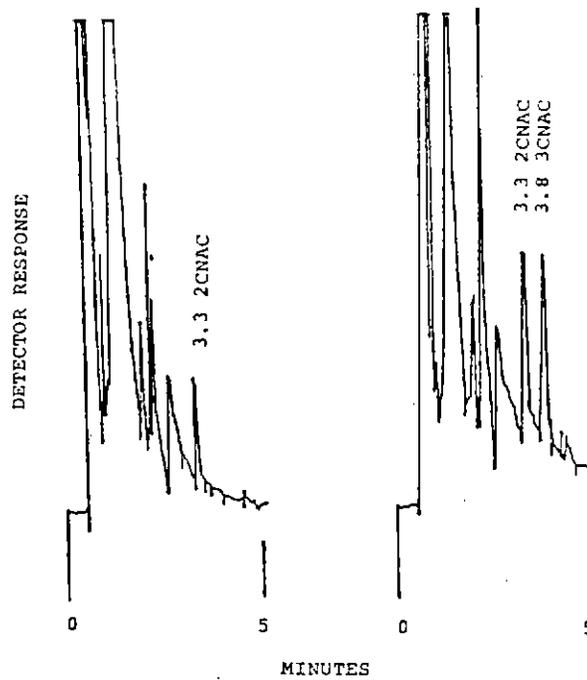


Figure 1. Electron capture gas chromatogram of a blank urine extraction (described in procedure) and a workers urine containing 10 ug/mL of 2CNAC (Rt=3.3) and 20 ug/mL of 3CNAC (Rt=3.8). Conditions were as follows. Injector: split ratio 1:50, Injector temperature: 300°C, Column: 0.25 mm x 15 m fused silica coated with methyl silicone, Column oven temperature program: 180° isothermal, Detector temperature: 300°C, Carrier gas flow: 2.3 mL/min.

3CNAC. Figure 2 shows the selected ion chromatogram of a subject exposed to DCP. The retention time of 2CNAC methyl ester is 10.33 minutes and 3CNAC methyl ester is 10.58 minutes. While not quantitated in these studies, small amounts of trans 3CNAC methyl ester may appear at 10.75 min. Figure 3 shows the mass spectrum of 3CNAC methyl ester. The 2CNAC methyl ester has similar abundances for ions 117 and 176 used in quantitation. Table I shows the operating characteristics of this assay by capillary GC-MS as compared to the previously used packed column GC-MS (2).

Table I. Operating characteristics of GC-MS Methods for Urinary 3C-NAC Determination

		Packed	Capillary
Linearity		0-30 ug/mL	0-30 ug/mL
Regression Coefficient		0.999	0.999
Sensitivity	117 ion	0.3 ug/mL	0.1 ug/mL
	176 ion	0.3 ug/mL	0.2
Specificity	117 ion	good	good
	176 ion	excellent	excellent
Coefficient of variation at 10 ug/mL		4%	8%

Biological monitoring. Air concentrations of DCP, urinary concentrations of 3CNAC and urinary activity of NAG are given for each subject in Figures 4, 5 and 6. Air concentrations of DCP varied from 0-10.3 ppm (0-47 ng/m<sup>3</sup>). The application of DCP accounted for >80% of the time of exposure during work hours. Mixing/loading, repair and break periods accounting for the remainder of the time.

Urinary concentrations of 3CNAC tended to rise and peak during or shortly after exposure. Lags between peak exposure and peak excretion were 0-16 hours. Urinary NAG activity did not seem to rise and fall with exposure to DCP or urinary excretion of 3CNAC, though it was abnormally elevated in two of three subjects at least once during the course of this pilot study.

Figure 7 shows that the 24 hour excretion of 3CNAC (mg) increased with rises in the daily cumulative conc\*time product for DCP. Also, the concentration of 3CNAC in the urine on the morning following exposure increases in rough proportion to the cumulative conc\*time product for DCP from the previous days (Fig. 8). (The correlations for evening urine concentration or excretion on the morning after (ug/mg C) were not as precise.)

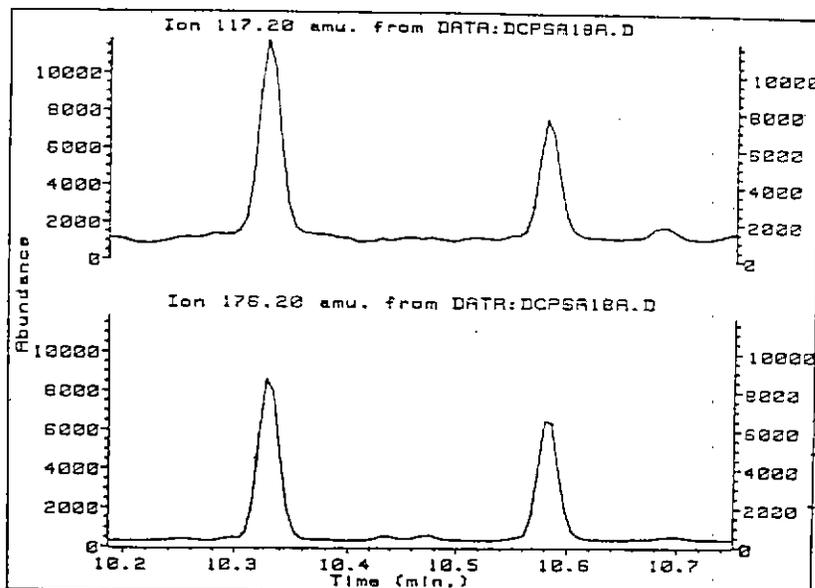


Figure 2. Selected ion chromatogram of subject exposed to DCP. 2CNAC (Rt=10.33) is 10 ug/mL and 3CNAC (Rt=10.58) is 10 ug/mL. Conditions in text.

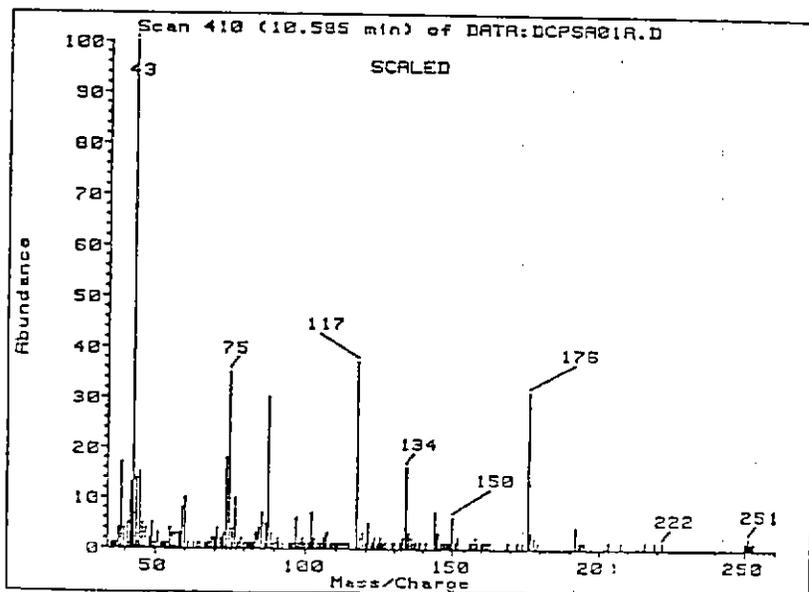


Figure 3. Mass spectrum of the 3CNAC peak at 10.58 minutes.

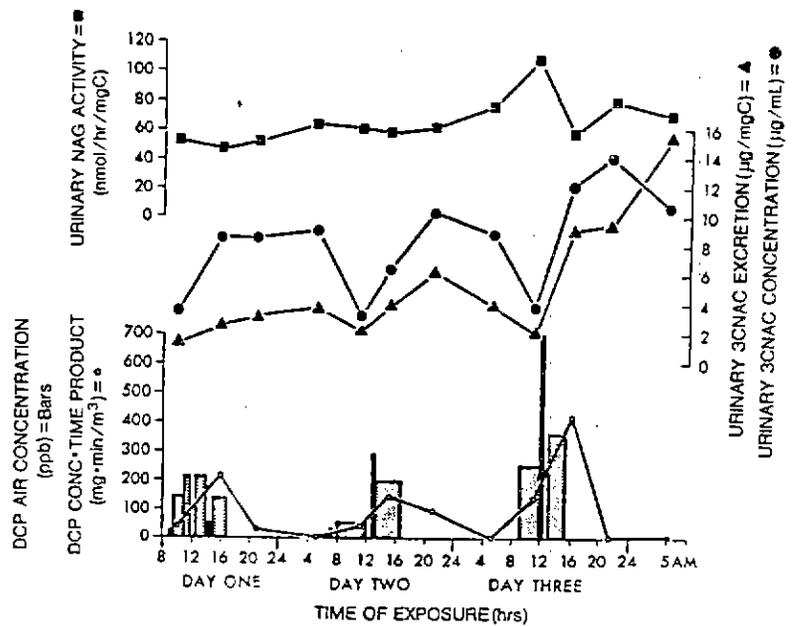


Figure 4. Subject #1. Air concentrations of DCP, urinary concentrations and excretion of 3CNAC, and urinary activity of NAG are given. mgC = milligram of creatinine. Conc\*time product is computed for each preceding exposure interval.

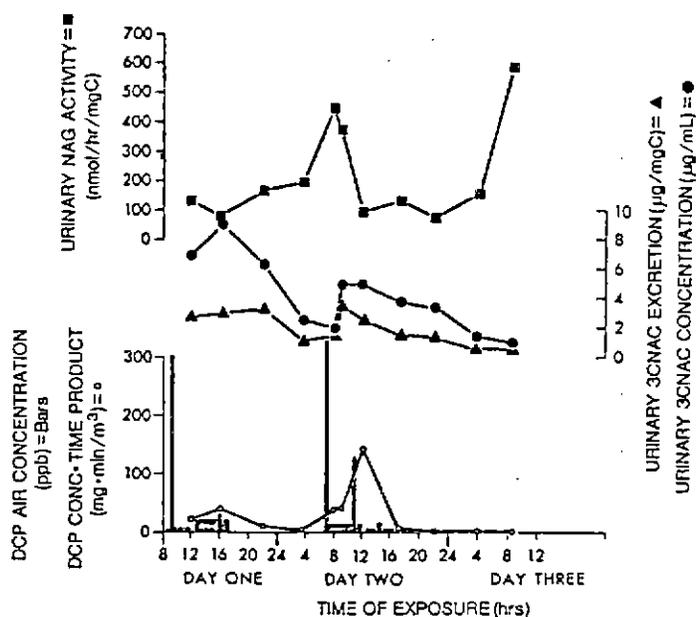


Figure 5. Subject #2. Air concentrations of DCP, urinary concentrations and excretion of 3CNAC, and urinary activity of NAG are given. mgC = milligrams of creatinine. Conc·time product is computed for each preceding exposure interval. Note change in scale for some measures as compared to Figures 4 and 6.

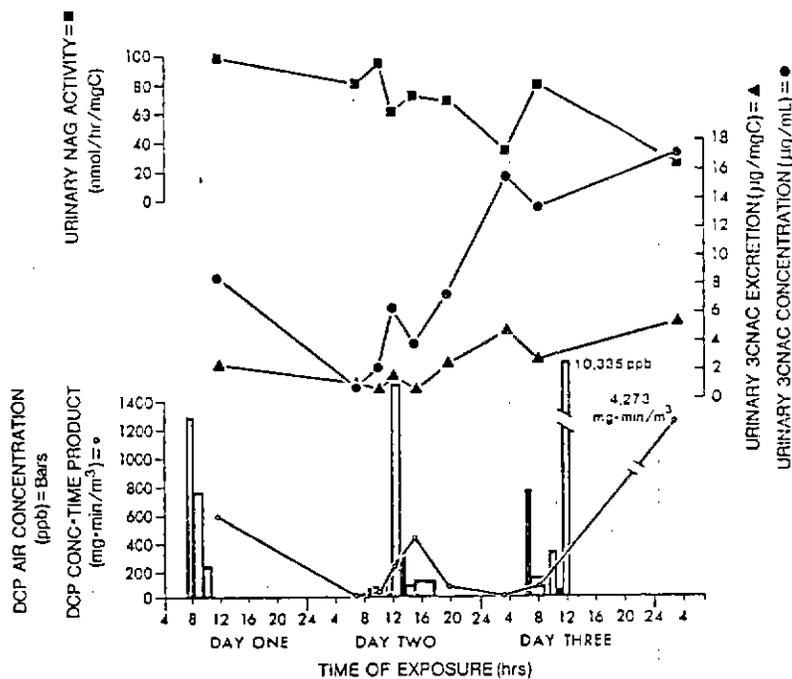


Figure 6. Subject #3. Air concentrations of DCP, urinary concentrations and excretion of 3CNAC, and urinary activity of NAG are given. mgC = milligrams of creatinine. Conc-time product is computed for each preceding exposure interval. Note change in scale for some measures as compared to previous Figures 4 and 5.

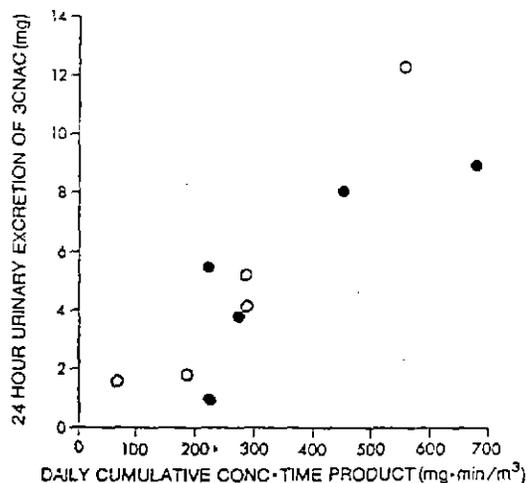


Figure 7. Plot of 24 hour excretion of 3CNAC versus cumulative 24 hr conc-time product for DCP air exposure. Data from subjects #1 and 2. ● = 1984 study of five 24 hr urine collections on five persons (from Ref. No. 2). ○ = Five 24 hr urine collections in this study from two persons.

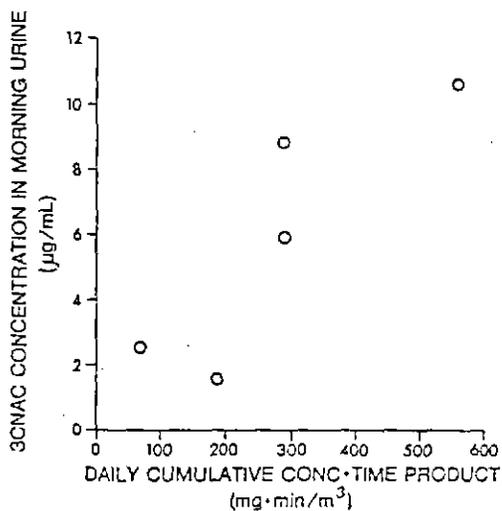


Figure 8. Plot of urinary concentration of 3CNAC from morning urine versus cumulative 24 hr conc-time product for DCP air exposure on preceding day.

Discussion

Urinary 3CNAC Procedures. Our prior attempts at capillary GC-MS methods have been several-fold less sensitive than GC-MS procedures in determining 3CNAC in urine samples. There are two likely reasons for this: 1) 3CNAC is polar and difficult to isolate with selectivity from other polar urinary acids (intermediary acids of metabolism and similar amino acids such as methionine); 2) the single chloro group on a polar molecule only slightly enhances the electron capture signal strength over other polar molecules. Recently, Onkenhout et al (1986) has used other detectors (nitrogen phosphorus, sulphur selective and mass selective) to detect the urinary metabolites in rats. We find that with capillary nitrogen selective gas chromatography of the 3CNAC in urine, the sensitivity is again limited due to endogenous urinary components. Onkenhout et al used different columns composed of methyl and phenyl groups to separate cis and trans 3CNAC. We have been able to separate cis and trans-3CNAC on methyl silicone columns under GC-MS conditions, but have not used trans 3CNAC as a marker for exposure since earlier animal studies indicated less was excreted in the urine (3). From qualitative observation of past chromatograms on humans, relatively little trans isomer is excreted compared to cis-3CNAC.

Our GC-MS approach has been to monitor both ions 117 and 176 m/e. An occasional human sample will have an interfering substance near the 117 ion peak, whereas the 176 ion gives routinely clean peaks. It would not seem likely that the 176 ion is more selective, since this ion is characteristic of any mercapturic conjugate if at that specific retention time. Monitoring more specific ions of lower abundance (e.g. molecular ion, 251) reduces sensitivity accordingly.

While intraassay precision was slightly worse when compared to the older packed column GC-MS (2), this is attributable to more complicated extraction in the capillary GC-MS procedure (3 steps vs. 6). This might be improved by omitting the evaporation - chlorbutane/water wash steps and choosing the above-mentioned more selective ions.

Biological monitoring. In field studies of an individual, replicate measures of personal air concentrations are difficult. Data from single personal air samplings are intrinsically "time averaged" for the period of collection. Therefore, a single high air concentration for a short period may bias the overall collection. In this pilot study of human exposure in the field, such air concentrations represent first approximations of potential exposure. The urinary concentrations of a metabolite of a pesticide are considered an estimate of actual and individual exposure. However, this is only true when relationships between absorption, distribution, metabolism and excretion are known with some certainty. Usually, inherent biovariability between individuals precludes

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establishing explicit relationships or limits. General relationships between exposure and excretion, exposure and effect or excretion and effect can be approached. In our pilot study, we have not established any certain relationships, but have uncovered those to investigate. Several practical considerations are evident as well.

In a larger study to follow, we intend to examine the relation between various expressions of exposure (peak concentrations, time weighted exposures and cumulative exposure) and the measures of excretion (ug/mL, ug/hour, ug/mg creatinine, total ug excreted). In this study, it can only be noted that excretion of metabolite does increase in response to DCP air exposure. This rise in metabolite excretion lags behind DCP peak exposure for a variable period of 0-16 hours. The few data points preclude determination of a precise relationship or pharmacokinetic analysis. However, the metabolite does not appear to accumulate and urinary amounts start to decrease just before the next exposure.

If urinary excretion of 3CNAC increases in proportion to what appears to be primarily a pulmonary exposure, then this may affirm the pulmonary route as the major route of exposure in workers. However, in unprotected workers dermal exposure and biologic variability would tend to confound this relationship. Stott and Kostl (1986) have shown that pulmonary uptake of DCP is 82% at 80 ppm in rats. Quantitative dermal absorption studies are not available. In actual work practice, milligrams of DCP can easily come into direct contact with the skin during repair and loading operations.

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Since urinary excretion of a metabolite such as 3CNAC is derived from all routes of absorption, urinary excretion may be more closely related to effects than air concentrations of DCP. Renal injury in animals following 3 ppm chronic inhalation exposure (13) is controversial since others have not seen alterations in histology or kidney weight following inhalation studies of 1-24 months at higher concentrations (50-90 ppm) (14,15). In part, this may be due to purer preparations of DCP used in recent experiments. Indeed, impurities causing direct mutagenicity can be removed from DCP preparations (8). These form in DCP preparations by autooxidation and may be a source of injury to humans, in which case DCP air measurements or urinary metabolite determinations would only serve as a marker for the impurity. However, DCP is an indirect mutagen following mono-oxygenase activation (8-11 and others). Thiols are protective of DCP mutagenicity in vitro (8,9). DCP will bind and deplete thiol stores in liver and kidney (12). Perhaps a subtle measure of DCP kidney toxicity, the significance of thiol depletion requires further investigation.

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In this pilot, NAG enzyme activity was chosen as a possible indicator of renal injury, should any occur at all with DCP exposure. NAG is a lysosomal enzyme released from renal

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tubular epithelial cells only during the period of acute injury. Price (1982) has reviewed its clinical relevancy. In rat models of transient renal tubular injury produced by maleic acid, NAG urinary activities begin to decrease within a few hours after injury has occurred (22). NAG excretion is considered to be increased only briefly after injury. We saw no clear correspondence between increased NAG urinary activity and excretion of metabolite in these three subjects. However, the fact that two of the three subjects had greater than normal (>100 nmol/hr/mg of creatinine) urinary activity at one point in our surveillance, requires that further investigation be made. It is reported that NAG urinary activity is constant for an individual (21). The interval to interval variability in these subjects suggests some external factor at work. It should be made clear that other unmeasured effects may contribute to elevated NAG activity such as underlying disease (other renal disease, hypertension), effects of medications (gentamicin, salicylates, phenacetin) or other chemical nephrotoxins such as heavy metals (see review by Price). Such variables were unknown in these subjects.

Of fundamental and practical concern are lessons learned from experience. Communication between worker and investigator should be clear. Missed portions of urine collections on subject 3 in this study precluded expression of urinary amounts of 3CNAC (total ug), but rather only creatinine corrected excretion. Subjects #1 and 2 excreted a total of 21.5 and 3.2 mg during the study period. But these amounts are misleading in this limited study, since pre-study exposure was not excluded. From the Figures (4-6), it is evident that subjects #2 and 3 were still excreting 3CNAC from a prior exposure at the beginning of the study. Baseline measurements are helpful when determining the magnitude of new metabolite excretion. Creatinine determinations should always be made in studies involving sequential or 24 hour urine collection. This allows not only to correct for urine flow dependency, but to assess the completeness of the collection.

Intervals of collection should coincide as much as possible with worker change in activity (end of day, end of application, lunch, repairs). Also, urine collections and air tube changes should coincide. These two recommendations enable easier data analysis.

From this pilot study, it was learned that NAG excretion during DCP exposure requires investigation and that 3CNAC excretion is not exactly concurrent with exposure. Collection of urine for sometime during and after (<24 hours) worker exposures may be representative of that earlier exposure.

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