Studies of the Toxic Interactions of Disinfection By-Products

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A large number and variety of compounds are formed in the process of chlorinating drinking water. The classes of compounds formed include trihalomethanes, haloacetic acids, haloacetonitriles, halophenols, and halopropanones. Many of the compounds have been shown to be toxic and are currently being further evaluated by the U.S. Environmental Protection Agency (EPA).

One group of the halopropanones found in chlorinated drinking water is the dichloropropanones. The toxicological properties of this group have not been well characterized. In addition, a number of investigators have shown that ketones potentiate the hepatotoxicity of haloalkanes. We conducted a series of studies to explore both the toxicity of the dichloropropanones and their potential interactions with a well-characterized haloalkane, carbon tetrachloride. A variety of toxicological and biochemical endpoints were used to evaluate the toxicity of the dichloropropanones and their interaction with CCl₄, including cytochrome P-450 concentration, reduced glutathione levels, pentane generation, serum enzyme activities, and histopathology.

Administration of 1,1-dichloropropanone (DCP) resulted in elevated serum enzymes associated with perportal necrosis. Glutathione levels were reduced by the administration of 1,1-DCP; pentane generation was not increased. When 1,1-DCP was given prior to CCl₄, the data were consistent with additivity. Administration of 1,3-DCP did not result in elevated serum enzymes, nor was there histopathologic evidence of necrosis. Glutathione levels and pentane generation in the 1,3-DCP-treated groups were the same as those of controls. Inhibition of the toxicologic effects of CCl₄ in a dose-related manner was observed when 1,3-DCP was administered prior to CCl₄.

Introduction

Since Rook (1) demonstrated the formation of chloroform and other chlorobrominated methanes resulting from the chlorination of water containing humic substances and inorganic bromine, a number of studies have confirmed and elaborated on the formation of halocompounds as by-products of the chlorination process (2–6). The classes of halogenated compounds that are formed include acids, alcohols, hydrocarbons (aromatic and nonaromatic), ketones, and aldehydes (5).

One group of ketones that is formed, but that has not been investigated toxicologically, is the dichloropropanones (DCP) (also known as dichloroacetones). Since many of the short-chained hydrocarbon contaminants of drinking water are known hepatotoxins (e.g., halomethanes), one purpose of these studies was to collect hepatotoxicity data on 1,1-dichloro-2-propanone (1,1-DCP) and 1,3-dichloro-2-propanone (1,3-DCP).

It is well recognized that aliphatic alcohols and carbon tetrachloride (CCl₄) interact (7–14). Reports in the literature also indicate that ketones and CCl₄ interact and that ketones and chloroform interact as well (16–19). Because the DCPs are ketones, a second purpose for conducting these studies was to examine the interactions between the DCPs and CCl₄.

Methods

CD-1 mice were used in these experiments and were obtained from Charles River (Portage, MI), held for a one-week quarantine period, and randomly housed, five per plastic cage, on hardwood chip bedding (Absorb-Dri, St. Regis Paper, Garfield, NJ). Purina Lab Chow and distilled water that had been carbon filtered and irradiated with ultraviolet light were available ad libitum. The room in which the animals were housed was maintained at 20–22°C with 40–60% relative humidity and 12-hr interval light-dark cycles. Doses of CCl₄ (reagent grade, Allied Chemical, Morrisstown, NJ) and/or 1,1-DCP (Aldrich, Milwaukee, WI) were administered using paraffin oil (Fisher Scientific, Fairlawn, NJ) as the vehicle, in constant volumes of 0.1 mL/10 g body weight. 1,3-DCP was administered in distilled water at the same constant volumes. Doses of CCl₄ used in the interaction studies were selected based on preliminary experiments indicating that the effects were observable.
and were in the beginning portion of the dose-response curve. The high doses of 1,1- and 1,3-DCP were the maximally tolerated doses.

For serum enzyme determinations, blood samples were collected by heart puncture from the right ventricle immediately after anesthesia with sodium pentobarbital (Nembutal). The blood was allowed to clot and then was centrifuged in a Beckman tabletop centrifuge (TI-JE) at 2400 g. Pasteur pipettes were used to transfer the sera to small vials that were then frozen at -70°C. At the termination of the study, serum enzyme activities (alanine aminotransferase [ALT], aspartate aminotransferase [AST], and lactate dehydrogenase [LDH]) were measured during contiguous runs using a CentrifilChem-400 high-speed centrifugal kinetic analyzer (Baker Instruments) and using identical reagent batches and controls. For quality control, all analytical runs included Dade Monitrol I and Monitrol II control sera.

To determine the level of in vivo pentane generation caused by a particular compound, the method of Frank et al. (20) was modified. Three fasted, male mice were gavaged with the appropriate dose of the compound(s) of interest and then immediately placed into a 500-cm³ oxygen-flushed desiccator. Soda lime, located at the bottom of the desiccator, removed respired carbon dioxide. The absorbed carbon dioxide was replaced by oxygen, available from a Mylar bag that was attached to the desiccator by tygon tubing. After the mice were in the closed system for 2 hr, two aliquots of air were taken using 10-cm³ gas-tight syringes. These samples were then injected into a Finnigan gas chromatograph (model 9503). The temperatures of the injector, the n-octane/ Porasil-C column (6 ft in length), and the flame ionization detector were 50°C, 22°C (room temperature), and 150°C, respectively. Gas flow rates were: helium, 25 cm³/min; hydrogen, 30 cm³/min; air 150 cm³/min. The standard curve was generated by evaporating a specific amount of liquid pentane in a known volume of room air. This stock pentane-air mixture was shaken, and aliquots of the gas were removed and placed in a second syringe having a total volume of 10 cm³. The resulting 10-cm³ mixtures were injected into the gas chromatograph. The data consisted of integrated values of the pentane peak.

Cytochrome P-450 was measured (24 hr after dosing) in microsomes (protein concentration was approximately 5–10 mg/mL) obtained by centrifuging the supernatant from an 8000g-for-10-min spin for 1 hr at 105,000 g. Using a double-beam spectrophotometer, 0.5 mL of microsomal suspension was diluted with 3 mL of a 0.05 M phosphate buffer (pH 7.4 at 22°C), a few crystals of sodium dithionite were added, and carbon monoxide was used to slowly gas the mixture for three minutes. The blank cuvette consisted of the diluted microsomal suspension.

The glutathione (GSH) assay was a modification of Hissin and Hilf's (81) photofluorometric methodology. After cervical dislocation, each experimental animal was perfused, via left ventricular heart puncture, with cold saline. The whole liver was weighed, trimmed to a 1-g piece, and then homogenized in a 0.1 M sodium phosphate–0.005 M ethylenediaminetetraacetic acid (EDTA) buffer at a pH of 8.0 at 37°C. After removing an aliquot for protein determination (Bio-Rad Standard Protein Assay, Richmond, CA), 25% m-phosphoric acid (m-PA) was added to precipitate proteins. The precipitate was then pelleted by centrifuging at 2500g for 10 min. The supernatant (0.05 mL) was diluted with 4.95 mL of the phosphate EDTA buffer. In a cuvette, 0.1 mL of this dilution was added to 1.8 mL buffer. Fifteen minutes after the addition of 0.1 mL o-phthalaldehyde, the fluorescence was measured at 420 nm after excitation at 350 nm. If mitochondrial levels of GSH were determined, a Sorvall RC-5B Refrigerated Superspeed centrifuge, with an SS-34 rotor, was used at 500g for 10 min to pellet the nuclear debris and unbroken cells. Subsequently, the supernatant was centrifuged at 2500g for 13 min. This mitochondrial pellet was resuspended in buffer and centrifuged a second time. The pellet was resuspended and an aliquot of the suspension was removed for protein determination. Protein was precipitated from the remainder of the sample by addition of m-PA, and processing was continued as previously described.

Experimental data were entered into and analyzed by the Statistical Analysis System (SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) for each parameter that had equal variance between treatment groups and normal distribution was conducted to test the null hypothesis that there was no response difference between groups. When the results of the ANOVA indicated an effect (p < 0.05), Tukey's multiple comparison test was used to detect differences (p < 0.05) between treatment groups. For parameters of unequal variances, nonparametric tests were applied (Kruskal-Wallis), and the medians were reported.

Results

Serum Enzyme Activities

The median values are reported in Tables 1 and 2 because as the enzyme values increased, so did the variances. Therefore, the appropriate statistical analysis was nonparametric (Kruskal-Wallis). Table 1 contains median enzyme values for mice exposed to 1,1-DCP and/or CCl₄, 1,1-DCP, when administered as above, caused an elevation in AST, ALT, and LDH at a dose of 0.25 mL/kg. As expected, CCl₄, when administered as above, caused a dose-related increase in all the measured enzymes. The data for the interaction of 1,1-DCP and CCl₄ are consistent with additive toxicity.

Table 2 contains median enzyme values for mice exposed to 1,3-DCP and/or CCl₄. No dose of 1,3-DCP (administered alone) in these acute studies caused an increase in AST, ALT, or LDH. A dose of 0.02 mL/kg CCl₄ consistently caused an increase in the three en-
Table 1. Clinical chemistry values (medians) for 1, 1-DCP and/or CCl₄.

| Dose       | 1,1-DCP, CCl₄, g/mL/kg | AST, IU/L | ALT, IU/L | LDH, IU/L |
|------------|-------------------------|-----------|-----------|-----------|
| Experiment 1 |                         |           |           |           |
| 0.00       | 0.00                    | 70 A      | 10 A      | 490 A     |
| 0.01       | 0.00                    | 80 A      | 10 A      | 420 A     |
| 0.05       | 0.00                    | 100 A     | 10 A      | 415 A     |
| 0.10       | 0.00                    | 75 A      | 25 A      | 325 A     |
| 0.25       | 0.00                    | 2670 CD   | 2590 BC   | 2575 B    |
| 0.00       | 0.02                    | 610 B     | 1500 B    | 3460 B    |
| 0.10       | 0.02                    | 2105 BC   | 5255 CD   | 10105 BC  |
| 0.25       | 0.02                    | 5520 D    | 7560 D    | 12465 C   |

*Both 1, 1-DCP and CCl₄ were administered separately in paraffin oil (0.1 mL/kg body weight) by oral gavage. 1,1-DCP was given approximately 4 hr before CCl₄. The mice were sacrificed 24 hr after the 1,1-DCP dose was administered.

*bMedians with the same letter (within the same experiment) are not statistically different. Significant differences (p<0.05) were determined by a Kruskal-Wallis test because variances were unequal. N = 12 for each group.

Table 2. Clinical chemistry values for 1,3-DCP and/or CCl₄.

| Dose       | 1,3-DCP, CCl₄, g/kg | AST, IU/L | ALT, IU/L | LDH, IU/L |
|------------|---------------------|-----------|-----------|-----------|
| Experiment 1 |                     |           |           |           |
| 0.00       | 0.00                | 60 A      | 20 A      | 345 A     |
| 0.10       | 0.00                | 60 A      | 30 A      | 405 A     |
| 0.00       | 0.01                | 75 A      | 20 A      | 355 A     |
| 0.00       | 0.02                | 580 B     | 2300 B    | 4025 B    |
| 0.10       | 0.01                | 60 A      | 20 A      | 330 A     |
| 0.10       | 0.02                | 380 B     | 1340 B    | 2605 B    |

*a,1-DCP and CCl₄ were administered separately in paraffin oil (0.1 mL/kg body weight). 1,3-DCP was administered in distilled water. All mice were dosed by oral gavage. The DCPs were given 2 hr before CCl₄ administration.

*bMedians with the same letter are not statistically different. Significant differences (p<0.05) were determined by a Kruskal-Wallis test.

Table 3. Pentane generation values for 1,1-DCP, 1,3-DCP, and CCl₄.

| Dose       | 1,1-DCP, g/kg | 1,3-DCP, g/kg | CCl₄, mL/kg | Pentane, mmole/kg/hr |
|------------|--------------|--------------|------------|----------------------|
| Experiment 1 |               |              |            |                      |
| 0.00       | 0.00         | 0.00         | 0.00       | 5 A                  |
| 0.1        | 0.00         | 0.00         | 0.00       | 5 A                  |
| 0.25       | 0.00         | 0.00         | 0.00       | 5 A                  |
| 0.01       | 0.01         | 0.00         | 0.00       | 5 A                  |
| 0.02       | 0.02         | 0.00         | 0.00       | 5 A                  |
| 0.01       | 0.01         | 1.0          | 0.00       | 5 A                  |
| 0.01       | 0.02         | 1.0          | 0.00       | 5 A                  |

Pentane Generation

Table 3 contains the pentane generation results of various combinations of 1,1-DCP, 1,3-DCP, and CCl₄. Very little pentane was generated in either control, 1,1-DCP- or 1,3-DCP-treated mice. The groups of animals exposed to CCl₄ showed an increase in the amount of pentane produced. However, the magnitude of this increase was not dose dependent, and neither 1,1-DCP nor 1,3-DCP caused pentane production in this experimental set-up. 1,3-DCP had an inhibitory effect on CCl₄ toxicity, as determined by pentane generation, in that pentane levels returned to control levels. These data support the clinical chemistry results.

Histology

No significant differences were observed between control mice and those exposed to 1,3-DCP. The mice exposed to 1,1-DCP showed evidence of perportal necrosis (a large number of dead hepatocytes around the portal triads). This condition was observed only in mice exposed to 0.25 mL/kg. The mice exposed to CCl₄ showed the expected signs of centrilobular necrosis (a large number of dead hepatocytes around the central vein). The lowest dose at which centrilobular necrosis was observed was 0.02 mL/kg.

In relation to the interaction studies between 1,1-DCP and CCl₄, both perportal and centrilobular necrosis was noted when 0.25 mL/kg 1,1-DCP was administered. The pathology report indicated that the results of the 1,3-DCP and CCl₄ interaction suggested that the "centrilobular necrosis may be slightly more severe in groups without 1,3-DCP than in the groups with 1,3-DCP."
Table 4. Cytochrome P-450 values for 1,1-DCP, 1,3-DCP, and/or CCl₄.

| Dose (mL/10g) | 1,1-DCP | 1,3-DCP | CCl₄ | P-450, nmole/g protein | N  |
|--------------|---------|---------|------|-----------------------|----|
| 0 ± 0.05     |         |         |      | 1.12 ± 0.05 A²       | 24 |
| 0 ± 0.05     | 0.01    |         |      | 0.98 ± 0.12 AB       | 12 |
| 0 ± 0.05     | 0.02    |         |      | 0.76 ± 0.08 B        | 9  |
| 0 ± 0.05     | 0.05    |         |      | 0.42 ± 0.05 C        | 6  |
| 0 ± 0.05     | 1.00    |         |      | 0.26 ± 0.08 C        | 6  |
| 0.01         |         |         |      | 0.89 ± 0.08 B        | 6  |
| 0.01         | 0.01    |         |      | 1.06 ± 0.04 A        | 6  |
| 0.01         | 0.02    |         |      | 1.20 ± 0.07 A        | 6  |
| 0.01         | 0.02    |         |      | 1.05 ± 0.10 A        | 6  |
| 0.01         | 0.01    |         |      | 1.07 ± 0.08 A        | 6  |
| 0.01         | 0.02    |         |      | 1.09 ± 0.15 A        | 6  |
| 0.02         | 0.02    |         |      | 0.69 ± 0.13 B        | 5  |

1,1-DCP and CCl₄ were administered separately in paraffin oil (0.1 mL/10 g body weight). 1,3-DCP was administered in distilled water. All mice were dosed by oral gavage. The DCPs were given 2 hr before CCl₄ administration.

²Medians with the same letter are not statistically different. Significant differences (p<0.05) were determined by a Kruskal-Wallis test.

Cytochrome P-450

Table 4 contains data on the cytochrome P-450 levels associated with various combinations of 1,1-DCP, 1,3-DCP, and CCl₄. A dose-related decrease in P-450 levels was observed in CCl₄-exposed mice. Neither 1,1-DCP nor 1,3-DCP affected the concentrations of P-450. In addition, neither 1,1-DCP nor 1,3-DCP appeared to interact with CCl₄.

GSH

Table 5 contains data on the GSH concentrations associated with 1,1-DCP, 1,3-DCP, and CCl₄ administration. The only compound that significantly decreased the GSH concentration was 1,1-DCP.

Table 5. Effects of 1,1-DCP, 1,3-DCP, or CCl₄ treatments on glutathione levels in hepatic homogenates and subcellular fractions of male mice.

| Treatment | Dosage mL/kg | N  | Whole homogenate: GSH (mg/g liver) | Mitochondrial fraction: GSH (μg/mg protein) | Postmitochondrial fraction: GSH (μg/mg protein) |
|-----------|--------------|----|-----------------------------------|---------------------------------------------|-----------------------------------------------|
| 1,1-DCP   | 0            | 12 | 1.80 ± 0.11                       | 4.52 ± 0.55                                  | 14.5 ± 1.32                                   |
|           | 0.01         | 6  | 1.72 ± 0.22                       | 4.82 ± 0.47                                  | 16.9 ± 1.87                                   |
|           | 0.05         | 5  | 1.45 ± 0.13                       | 4.79 ± 0.90                                  | 11.6 ± 2.32                                   |
|           | 0.1          | 6  | 1.17 ± 0.09²                      | 4.66 ± 0.76                                  | 8.0 ± 1.09b                                   |
| 1,3-DCP   | 0.01         | 5  | 0.56 ± 0.10²                      | 0.90 ± 0.25a                                  | 3.8 ± 0.52b                                   |
|           | 0.02         | 8  | 1.87 ± 0.39                       | 4.70 ± 0.97                                  |                                               |
| CCl₄      | 0            | 12 | 0.78 ± 0.24                       | 4.47 ± 0.80                                  |                                               |
|           | 0.2          | 6  | 1.46 ± 0.17                       | 4.51 ± 0.87                                  | 14.0 ± 2.06                                   |
|           | 0.5          | 6  | 1.16 ± 0.21                       | 3.21 ± 0.85                                  | 11.2 ± 3.37                                   |
|           | 1.0          | 6  | 1.43 ± 0.25                       | 3.18 ± 0.44                                  | 15.1 ± 3.05                                   |
|           | 2.0          | 6  | 1.78 ± 0.15                       | 2.65 ± 0.27                                  | 19.4 ± 2.25                                   |

* Both DCA and CCl₄ were administered separately in paraffin oil (0.1 mL/10 g body weight) by oral gavage 2 hr before sacrifice. Values represent the means ± SE from 10 mice.

Discussion

During determinations of the appropriate doses of 1,1-DCP and 1,3-DCP to administer when measuring the effects of these compounds on serum enzyme activities, it became clear that each was, by definition, very to extremely toxic (the LD₅₀ of 1,1-DCP was 250 mg/kg and the LD₅₀ of 1,3-DCP was 25 mg/kg; unpublished data). This finding is of interest because the LD₅₀ for mice exposed to 2-propanone has ranged from 4000 to 8000 mg/kg (25) (slightly to moderately toxic). Therefore, chlorination of 2-propanone dramatically increases its toxicity.

Although chlorination makes 2-propanone more toxic, the locations of the two chlorine substituents are important to the type of toxicity observed. No liver toxicity was associated with 1,3-DCP. However, 1,1-DCP was hepatotoxic as indicated by both clinical chemistry evaluations and histopathology. Serum enzymes, indicative of necrotic cells, were elevated. The histopathological analysis of liver sections indicated periportal necrosis associated with exposure to the 1,1-DCP isomer. The other parameter affected by 1,1-DCP, but not by 1,3-DCP, was the level of GSH, which was decreased. The necrosis caused by 1,1-DCP was not observed until 1,1-DCP reduced GSH to 30% of control levels. GSH serves an important role in the liver as a defense against chemicals binding to cellular macromolecules (24). For example, Thor et al. (25) showed that chlorobenzene toxicity dramatically increased when GSH levels were decreased by prior treatment with diethyl maleate. Therefore, the necrotic effect of 1,1-DCP seems to be blocked by GSH until GSH is reduced to a level that is no longer protective. It does not appear that 1,1-DCP causes the necrosis as a result of lipid peroxidation (as evidenced by the lack of pentane production).

The toxicologic relationship between 1,1-DCP and CCl₄ is additive. The prior depletion of GSH by 1,1-DCP would not be expected to cause a synergistic in-
crease in the extent of necrosis caused by a compound that is metabolized to an organic free radical (i.e., CCl₄) (26). Therefore, the additive relationship is consistent with available data concerning the biochemical effects of these compounds.

The toxicologic relationship between 1,3-DCP and CCl₄ is clearly one that is less than additive. This conclusion is based on three different experimental paradigms: serum hepatic enzyme, pentane generation, and histopathology. The information collected by these experimental designs is not sufficient to determine the mechanism by which 1,3-DCP interferes with the expression of CCl₄ toxicity. However, without the "lethal cleavage" of chlorine to form the trichloromethyl radical (27), CCl₄ may not cause its characteristic toxicologic changes. Although one might expect that 1,3-DCP destroys cytochrome P-450, the data in Table 4 show that 1,3-DCP does not destroy the cytochrome P-450 complex. There are several possibilities for where the interference could be occurring: the absorption of CCl₄ could be reduced; there could be competitive inhibition for the mixed-function oxidase system; there may be a shift in CCl₄ metabolism; and/or there may be increased scavaging of free radicals that are formed after homolytic cleavage of one of the chlorine substituents in the parent compound. A variety of experiments need to be completed before definitive statements can be made about the locus of interference.

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