Conversion of Trichloroacetic Acid to Dichloroacetic Acid in Biological Samples

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Abstract

Trichloroethylene (TCE) has been identified as an environmental contaminant in groundwater. Trichloroacetic acid (TCA), dichloroacetic acid (DCA), chloral hydrate (CH), trichloroethanol (TCOH), and trichloroethanol glucuronide (TCOG) have been identified as metabolites of TCE. Studies have shown that TCA and DCA are toxicologically significant metabolites that can induce liver tumors in B6C3F1 mice. Methods for the analysis of these metabolites are important for conducting pharmacokinetic studies. In this study, TCA and DCA were derivatized to their methyl esters by dimethyl sulfate under acidic conditions and analyzed by gas chromatography with electron capture detection. In developing a method for esterifying TCA and DCA, the conversion of TCA to DCA was observed in freshly drawn blood upon the addition of acid. After blood was drawn from the animals, the amount of TCA converted to DCA by the addition of acid decreased with time. This conversion could be prevented by freezing blood samples overnight prior to the addition of acid. Further experiments demonstrated that this activity could be restored by the addition of dithionite to inactive blood samples or the addition of dithionite to methemoglobin prior to the addition of acid. The results reported here show that reduced hemoglobin may be involved in the acid-catalyzed conversion of TCA to DCA.

Introduction

Trichloroethylene (TCE), a widely used degreasing and cleaning solvent, is an environmental contaminant commonly found in groundwater. Exposure to TCE is of concern because it has been found to be a rodent carcinogen (1). TCE is reported to be metabolized to chloral hydrate (CH), trichloroacetic acid (TCA), dichloroacetic acid (DCA), trichloroethanol (TCOH), and trichloroethanol glucuronide (TCOG) (2,3). DCA and TCA, like TCE, cause liver tumors in mice (4,5).

Analytical methods have been developed to analyze biological samples for TCE and its metabolites (6–9). Early colorimetric procedures used Fujiwara’s method to analyze urine for TCA and TCOH (7). Later, more sensitive gas chromatographic (GC) methods analyzed urine for TCA, TCOH, and TCOG (8). Urine was analyzed with and without sulfuric acid hydrolysis for TCOH and TCOG, and TCA was extracted into diethyl ether and derivatized to its methyl ester. Headspace GC methods have been used when analyzing whole blood for TCA, TCOH, and TCOG (9). Blood was acidified with concentrated sulfuric acid to convert TCOG to free TCOH, and dimethyl sulfated esterified TCA to its volatile methyl ester. Here we report new findings regarding the conversion of TCA to DCA in freshly drawn whole blood samples. This conversion occurs in the presence of acid and reduced hemoglobin. Previous methods have recognized the need to prevent the conversion of TCE metabolites. Breimer et al. (9) used lead acetate to prevent the enzymatic conversion of CH to TCOH in blood during incubation of samples for headspace analysis. They also reported that sulfuric acid will prevent the conversion of CH to TCOH by erythrocytes. Their recommendation is that blood samples be immediately mixed with lead acetate or sulfuric acid. Other methods have treated biological samples with sulfuric acid in order to extract DCA and TCA into an organic solvent (3,10). It is known that the addition of acid will affect TCOH concentrations because acid will hydrolyze TCOG to free TCOH (9,11). Therefore, acid-treated samples analyzed for TCOH will yield total TCOH because blood and urine have free and conjugated TCOH (10). Our experiments show that blood levels of TCA and DCA are also affected by the addition of acid. This metabolite interconversion occurs in freshly drawn blood that has been acidified with sulfuric acid or hydrochloric acid for the extraction and derivatization of TCA and DCA to their methyl esters.

Materials and Methods

Laboratory animals

Laboratory animals were obtained from Charles River Laboratories (Kingston, NY). Male B6C3F1 mice weighing 17–23g (6–7 weeks old) were used for this study. Animals were ex-
posed to 600 ppm TCE via inhalation for 4 h, and sacrificed by CO₂ asphyxiation. Unexposed mice were also sacrificed by CO₂ asphyxiation for control tissue samples.

**Chemicals and equipment**
TCE (99+% purity), TCA (99+% purity), DCA (99+% purity), 2,2-dichloropropionic acid (94% purity), methyl dichloroacetate (99+% purity), methyl trichloroacetate (99% purity), and dimethyl sulfate (99+% purity) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Lead acetate was obtained from Mallinckrodt (Paris, KY). Sulfuric acid, hydrochloric acid, and hexane were obtained from Fisher Scientific (Fair Lawn, NJ). Deferoxamine mesylate (DFA), bovine albumin (fraction V), and hemoglobin (human, rat, and mouse) were obtained from Sigma Chemical Co. (St. Louis, MO). The hemoglobin was in the form of methemoglobin. Sodium trichloroacetate (97%), reagent-grade sodium dithionite (sodium hydrosulfite), sodium nitrite, and ferrous sulfate were also obtained from Aldrich Chemical Co. A Haake-Buchler (Saddlebrook, NJ) vortex evaporator was used to vortex mix and heat samples. Samples were analyzed on a Hewlett Packard (Avondale, PA) 5890 GC with a Hewlett Packard 7673A liquid autosampler. The chromatograph was equipped with an electron capture detector (63Ni, 10 mCi) and a 30-m x 0.53-mm Supelco Wax 10 column (Bellefonte, PA). Chromatographic conditions were: injector, 175°C; initial temperature, 70°C; initial time, 15 min; heating rate, 15°C/min; final temperature, 190°C; final time, 5 min; and detector temperature, 300°C. The carrier and make up gas was 5% methane in argon, and the carrier flow rate was 6 mL/min.

**Assay procedures**
Samples were analyzed for DCA and TCA by modification of a method by Maiorino et al. (12). To prevent the conversion of TCA to DCA in freshly collected blood, samples were frozen overnight at -20°C prior to analysis. After thawing, a 0.1-mL sample of blood was placed in a 2-mL vial with 0.1 mL water, and 0.1 mL 2,2-dichloropropionic acid (10 µg/mL, internal standard). The vials were placed on ice and allowed to cool for 30 min. Concentrated sulfuric acid (0.5 mL) and a 0.1-mL aliquot of dimethyl sulfate were added. The vials were capped and vortex mixed on a shaking tube and stored at 4°C in a refrigerator (this sample was extremely hemolyzed); old human blood and dithionite; plasma drawn from centrifuged fresh mouse blood; plasma drawn from fresh mouse blood spiked with 20 µg/mL neutral sodium trichloroacetate that was allowed to stand for 30 min prior to separation by centrifugation; an aqueous solution of methemoglobin (150 mg/mL); and methemoglobin and dithionite. Additionally, the effects of deferoxamine (DFA), a known Fe(II) chelator, were examined. Two different addition sequences were made using blood, which was frozen overnight, from control animals. A 0.1-mL aliquot was combined with 0.1 mL of 2,2-dichloropropionic acid (10 µg/mL, internal standard) and a 0.1-mL aliquot of an aqueous standard of DCA and TCA. The standards were derivatized and analyzed as previously described.

The recovery of DCA and TCA is a function of both the reaction efficiency of the dimethyl sulfate derivatization and the extraction efficiency of the methyl esters into hexane. The reaction efficiency was obtained by comparing the areas of DCA and TCA derivatized and extracted from spiked blood samples with the area of blood samples spiked with authentic methyl esters of DCA and TCA and extracted into hexane. The extraction efficiency was obtained by comparing the area of authentic methyl esters of DCA and TCA extracted from spiked blood samples into hexane with the areas of unextracted methyl esters in hexane.

The following protocol was used to illustrate the dependency of the conversion of TCA to DCA on reduced hemoglobin. Into small glass centrifuge tubes were placed 100 µL each of neutral aqueous sodium trichloroacetate solution (20 µg/mL), the internal standard solution, the sulfuric acid solution (10%), and either water, sodium dithionite (1 M), or sodium nitrite (1 M). The test matrix (100 µL) was then added to the tubes. The contents were briefly vortex mixed and allowed to stand at room temperature for approximately 30 min. The samples were then processed as above and analyzed for a change in the TCA to internal standard ratio as a result of TCA dechlorination to DCA. Specific quantitation of TCA and DCA was not performed. Aqueous bovine albumin (100 mg/mL) served as a proteinaceous negative control. The results of the other matrices were referred to this matrix. The test matrices were as follows: freshly drawn, heparinized mouse blood; fresh mouse blood and dithionite; fresh mouse blood and nitrite; 8-month-old human blood drawn into a heparin tube and stored at 4°C in a refrigerator (this sample was extremely hemolyzed); old human blood and dithionite; plasma drawn from centrifuged fresh mouse blood; plasma drawn from fresh mouse blood spiked with 20 µg/mL neutral sodium trichloroacetate that was allowed to stand for 30 min prior to separation by centrifugation; an aqueous solution of methemoglobin (150 mg/mL); and methemoglobin and dithionite. Additionally, the effects of deferoxamine (DFA), a known Fe(II) chelator, were examined. Two different addition sequences

![Figure 1](https://academic.oup.com/jat/article-abstract/20/4/236/838518)
were used. In the first procedure, DFA was added to blood prior to the addition of the blood to the tubes containing TCA in sulfuric acid. This was designated as “blood + DFA a”. In the second procedure, neutralized TCA was mixed with fresh blood prior to the addition of DFA to blood, which was added to sulfuric acid. This was designated “blood + DFA b” and more closely resembles the situation of an actual sampling protocol. The activity of an aqueous solution of iron(II) sulfate was also examined. Using the above protocol, concentrations of 0, 2.5, 5, 10, 25, and 50 mM iron(II) sulfate were tested.

Results

DCA, TCA assay

The assay developed for DCA and TCA had a limit of detection of 1.0 μg/mL for the analysis of 0.1-mL blood samples. This was based on a signal-to-noise ratio greater than 3. The on-column limit of detection for the methyl esters of DCA and TCA was estimated at 50 pg, which was also based on a signal-to-noise ratio greater than 3. The method produced quadratic standard curves for DCA and TCA as shown in Figure 1. Typical curves for DCA and TCA were: \( y = -0.0510x^2 + 1.836x + 0.0484 \) (\( r^2 > 0.999 \)), and \( y = -0.0203x^2 + 5.93x + 0.0808 \) (\( r^2 > 0.999 \)), respectively. A chromatogram of DCA and TCA extracted and derivatized from blood spiked with 10 μg/mL of each is shown in Figure 2. The retention times of TCA, DCA, and DPA (internal standard) were 8.0, 9.1, and 5.1 min, respectively. The reaction efficiency of the dimethyl sulfate derivatization averaged 75% (\( n = 12 \)), and the extraction efficiency averaged 67% (\( n = 12 \)). The extraction and reaction efficiencies were measured in duplicate at 5, 25, and 50 μg/mL for both DCA and TCA. The precision of this method was evaluated in whole blood. The intraday variability for DCA and TCA had an average coefficient of variation of 7, 4, and 4% at 5, 25, and 50 μg/mL, respectively (\( n = 7 \)). The interday variability for DCA and TCA had an average coefficient of variation of 8, 8, and 7% at 5, 25, and 50 μg/mL, respectively (\( n = 6 \)).

Conversion of TCA to DCA

Fresh control blood was collected from mice, and 0.1-mL aliquots were placed into tubes, kept at room temperature, that contained 0.1 mL of 33 μg/mL neutral TCA (20 nmoles). At time intervals of 0, 1, 5, 10, 15, 30, and 60 min, 0.1 mL of 10% sulfuric acid was added. Three hours after the blood was taken, the samples were derivatized with dimethyl sulfate and analyzed. The results, which are shown in Figure 3, suggest that TCA is converted to DCA by the addition of 10% sulfuric acid to freshly drawn blood. The conversion of TCA to DCA also occurred when 15% hydrochloric acid, and other concentrations of sulfuric acid (5%, 25%, 50%, and 100%) were added to fresh blood (data not shown). The data shown in Figure 3 represent an average of four experiments in which TCA in fresh mouse blood was converted to DCA under acidic conditions with 10% H₂SO₄. The amount of TCA converted to DCA varied from 20% to 70%. However, the sum of DCA and TCA remained constant over the course of the experiment (20 ± 0.9 nmoles).

An aliquot (0.1-mL) of fresh blood from a control animal was added to an ice-cold vial that contained both TCA and 20% lead
acetate. Addition of 0.1 mL 10% sulfuric acid converted 80% of the TCA to DCA. However, if a TCA-spiked blood sample was frozen overnight at -20°C prior to analysis, TCA was not converted to DCA with the addition of 10% sulfuric acid. In this sample, the TCA concentration was consistent with the expected value. The addition of sulfuric acid to fresh mouse plasma spiked with TCA did not convert any TCA to DCA.

To demonstrate the effect of treating blood samples from exposed mice with acid, mice were exposed to 600 ppm TCE for 7 h. Following exposure, individual mice were sacrificed and blood samples were obtained at 0.08, 0.25, 0.5, 0.75, 1.5, 3, 5, 7, 9, and 18 h. Blood samples were collected at each time point and immediately split into two portions. One portion was put into an equal volume of 10% sulfuric acid and kept on ice; the second was combined with an equal volume of distilled water and also kept on ice. After collection, samples were stored at -20°C for 24 h and then analyzed for DCA and TCA. As shown in Figure 4, the samples immediately combined with acid contained DCA and TCA. Samples that were not combined with 10% sulfuric acid had higher concentrations of TCA and no DCA.

Table I summarizes the ability of various matrices to catalyze the conversion of TCA to DCA. The loss of TCA is expressed relative to the bovine albumin control in which no conversion of TCA was expected and none was observed. Reduced blood samples (i.e., those to which dithionite had been added) were all active in catalyzing this reaction, even 8-month-old hemolyzed blood and reduced, purified hemoglobin. Fresh blood was partially active, whereas fully oxidized bloods, either the 8-month-old blood sample or the fresh blood to which nitrite had been added, as well as the plasma samples, had no activity towards dechlorination of TCA. There was no difference between the TCA content of freshly separated plasma spiked with TCA and that of whole blood that had been spiked and allowed to stand for 30 min prior to plasma separation. Methemoglobin had no activity towards the dechlorination of TCA, whereas methemoglobin with dithionite (reduced hemoglobin) dechlorinated TCA extensively. There was a difference in the effect of DPA depending on whether it was added to blood containing TCA (blood + DFA b) or to the blood prior to addition to the TCA in sulfuric acid (blood + DFA a). The latter inhibited the conversion of TCA to DCA and the former did not. Finally, none of the aqueous solutions of Fe(II) were capable of catalyzing the dechlorination of TCA.

Discussion

This assay was developed for the determination of DCA and TCA in biological samples. However, the analysis of DCA and TCA in blood is complicated by an artifactual conversion of TCA to DCA. The conversion of TCA to DCA in fresh blood is an important concern for the analysis of these metabolites of TCE. Published methods for the analysis of TCA (7,9,10) in biological fluids routinely acidify samples with strong acid. This step is necessary in order to extract acids such as DCA (pK<sub>a</sub> 1.3) and TCA (pK<sub>a</sub> 0.7) into an organic solvent for methylation. Similar derivatization reactions developed for trifluoroacetic acid, such as the formation of methyl esters with N,N-dimethylformamide dimethylacetal (13) and derivatization with ethereal phenylmethanone (14), adjust biological samples to low pH values for the same reason. An alternative method for the derivatization of TCA is needed, and there are several possibilities: One method would be to extract and derivatize plasma. This would eliminate any conversion catalyzed by reduced hemoglobin. Conversion of TCA to DCA can be prevented by freezing blood samples at -20°C overnight before adding acid for derivatization. Freezing hemolysed red blood cells and facilitates the oxidation of Fe(II) to Fe(III). Blood samples may be treated with sodium nitrite, which will oxidize Fe(II) to Fe(III). It may also be possible to derivatize TCA in a biological sample by adding base, lyophilizing to remove water, and dissolving the residue in an organic solvent such as methanolic BF<sub>3</sub> for derivatization.

Our work has shown that the acidification of fresh whole blood samples can lead to erroneous results. This has been demonstrated to occur in vitro with fresh blood spiked with TCA and also with fresh blood obtained from mice exposed to TCE. Plasma proteins do not appear to be responsible for the conversion of TCA to DCA because the reaction does not occur in fresh plasma from mice. The finding that pretreating blood with lead acetate does not prevent the TCA to DCA conversion indicates that the conversion may not be due to enzymatic...
activity. The necessary dependence on reduced hemoglobin is clearly evident in the data presented in Table I. Only fresh blood samples and samples that included dithionite, an Fe(III) reducing agent, dechlorinated TCA. The data shown in Figure 3 demonstrate that the ability of fresh blood to convert TCA to DCA decreases with time. This may be attributed to the loss of reduced hemoglobin in blood allowed to stand at room temperature. The purified reduced hemoglobin data, shown in Table I, indicate that the hemoglobin component of the blood is the active constituent. The fact that no difference was observed between the spiked plasma sample and plasma separated from spiked blood indicates that this conversion only takes place upon acidification in the presence of the hemoglobin and that TCA is stable in blood at physiologic pH, which is an important consideration in sample handling.

DFA inhibited the dechlorination of TCA only when it was added to blood prior to the addition to the TCA in sulfuric acid (blood + DFA a). When DFA was added to blood already containing TCA (blood + DFA b), DFA failed to inhibit the dechlorination of TCA. The latter more closely resembles actual sampling situations; hence, it can be concluded that the addition of DFA would not prevent the conversion of TCA to DCA. However, in the absence of TCA, DFA is able to sequester the iron from the later, addition of TCA. Finally, the activity of aqueous solutions of Fe(II) was assessed. Concentrations ranging from 0 to 50 mM were inactive towards dechlorination of TCA. This indicates that for the electron transfer reaction to occur, the iron must be complexed, either with sulfur, as in ferredoxin, or with extensively delocalized nitrogens, as in the pyrrolic porphyrin ring structure of hemoglobin.

Similar reactions have been previously reported. Cammack et al. (15) reported that ferredoxin, an iron-sulfur containing protein involved in photosynthesis, was capable of photochemically reducing TCA to DCA using visible light. It was found that the ferredoxin iron had to be maintained in the reduced state with dithionite for the reaction to occur. The authors speculated that the iron-sulfur clusters were the origin of the reducing electrons. Rusling and co-workers (16) found that TCA could be reductively dehalogenated by Vitamin B12, a transition metal complex of cobalt. This reaction occurred at pH 3 and was found to be spontaneous when Vitamin B12 was in its reduced form. In addition, Manno et al. (17) and Ferrara et al. (18) have reported that human hemoglobin was capable of reductive activation of carbon tetrachloride. Their system was anaerobic, and again, the reduced form of the iron was maintained with dithionite.

Conclusion

The results reported here show that it is critically important to evaluate the ability of metabolites to interconvert during analytical procedures, particularly when collecting data for pharmacokinetic analysis and assessing the metabolite(s) of TCE responsible for its carcinogenicity. Our data has shown that the addition of acid to fresh blood samples can convert TCA to DCA. Blood samples need to be frozen overnight at −20°C prior to the addition of acid for DCA–TCA analysis.

Acknowledgments

This research was funded by the Strategic Environmental Research and Development Program and partially under U.S. Department of Defense Contract F33615-90-C-0532 at the Armstrong Laboratory Toxicology Division, Wright-Patterson Air Force Base, Ohio. Reprints of this article are identified by Armstrong Laboratory, Wright-Patterson Air Force Base, Ohio as AL/OE-TR-1996-0074. The authors wish to thank Harold F. Leahy and Peggy Parish for their excellent technical assistance. The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Laboratory Animal Resources, National Research Council, DHHS, National Institute of Health Publication No. 86-23, 1985, and the Animal Welfare Act of 1966, as amended.

References


