ENTEROHEPATIC RECIRCULATION OF TRICHLOROETHANOL GLUCURONIDE AS A SIGNIFICANT SOURCE OF TRICHLOROACETIC ACID

Metabolites of Trichloroethylene


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(Received April 5, 1996; accepted January 9, 1997)

ABSTRACT:

Trichloroacetic acid (TCA) is a metabolite of trichloroethylene (TRI) thought to contribute to its hepatocarcinogenic effects in mice. Recent studies have shown that peak blood concentrations of TCA do not occur until ∼12 hr after an oral dose of TRI; however, blood concentrations of TRI reach a maximum within 1 hr and are non-detectable after 2 hr. The objective of this study was to examine quantitatively enterohepatic recirculation of trichloroethanol (TCEOH) and TCA as a possible mechanism responsible for the delayed production of TCA. Jugular vein, duodenum, and bile duct-cannulated Fischer 344 rats were used, with the collection of blood, bile, urine, and feces samples after intraduodenal and intravenous dosing of animals with TRI, TCEOH, and TCA. Samples were analyzed by GC for TCA, total TCEOH, and free TCEOH. The results show that, after an intravenous dose of TCEOH (100 mg/kg), 36% of the TCEOH in blood is attributable to enterohepatic recirculation. With the same treatment, 76% of the TCA in blood is attributable to enterohepatic recirculation of metabolites. Peak concentrations of total TCEOH in bile, after an intraduodenal dose of TRI, are over 5 times higher than peak concentrations of total TCEOH in systemic blood. Peak concentrations of TCEOH glucuronide in bile are ∼200 times higher than peak concentrations of TCEOH glucuronide in systemic blood.

TRI1 (1,1,2-TRI) is a commonly used organic solvent and degreaser. It is widely used for adhesives and lubricants and also as a low-temperature heat transfer fluid, vapor degreaser, and spot remover in the textile industry (1, 2). In the past, it has also been used as a general anesthetic for surgical, dental, and obstetrical procedures in medical practice (3). As a result of this widespread use, TRI has become a common environmental contaminant. It has been found in surface water, ground water, ambient air, and soil. It has been found in drinking water, from ground water supply sources, tested in the United States (4).

Three metabolites of TRI have been shown capable of inducing liver tumors in mice, trichloroacetaldehyde, TCA, and DCA (5–8). Of these carcinogenic metabolites, TCA production results in the largest concentrations in blood. To provide an appropriate base for estimating risks from TCA to humans, it is important to understand factors that control blood concentrations of TCA.

Templin et al. (9) demonstrated that TRI is rapidly absorbed from the gastrointestinal tract, reaching a maximum before 1 hr, then is substantially eliminated from the blood within 2 hr. However, the increase in concentration of TCA in blood after an oral dose of TRI does not peak until ∼10–12 hr. This delay in TCA production suggests the existence of a reservoir for TRI or intermediate metabolites that undergo a slower conversion to TCA.

The primary objective of this study was to evaluate quantitatively the hypothesis that enterohepatic recirculation of TCEOH glucuronide and/or TCA significantly contributes to the formation and maintenance of TCA concentrations in blood. Figure 1 shows a schematic representation of the oxidative pathway of TRI metabolism in which enterohepatic recirculation is specifically considered.

Materials and Methods

Chemicals. Certified A.C.S.-grade TCEOH (99%, Aldrich Chemical Co., Milwaukee, WI), TRI (Fisher Scientific, Fair Lawn, NJ), and Tween 80 (Sigma Chemical Co., St. Louis, MO) were used in preparation of dosing solutions. TCEOH (99%, Aldrich Chemical Co.), DCA (Sigma Chemical Co.), and TCA (Sigma Chemical Co.) were used in the preparation of standard curves. Sodium DCPA (90%, Aldrich Chemical Co.) was used as the internal standard for gas chromatographic analysis. 1,2-Dichlorobenzene (Aldrich Chemical Co.), absolute alcohol (Midwest Grain Products Co., Atchison, KA), sodium chloride (J. T. Baker, Inc., Phillipsburg, NJ), isopropyl ether (99%, Aldrich Chemical Co.), and N-ethyl-N-nitrosoguanidine (Aldrich Chemical Co.) were used as reagents. Diaoethene, used in DCA and TCA derivatization, was prepared from N-ethyl-N-nitrosoguanidine after Aldrich Technical Information Bulletin Number AL-121. β-Glucuronidase (Helix pomatia, 416,800 units/g; Sigma Chemical Co.) was used for the hydrolysis of glucuronidated TCEOH in determining total TCEOH present in samples. All water used for analysis and for preparing dosing solutions was deionized and double distilled.

Animals. Male F-344 rats, weighing ∼300 g, were purchased from Hilltop Laboratory Animals, Inc. (Scottdale, PA). Cannulation surgery was performed by Hilltop Laboratory Animals, Inc., with light ether used to anesthetize the animals before surgery, and each animal held for a period of 2 days after surgery to ensure full recovery before shipping. Bile ducts were cannulated in two locations (i.e., immediately after the exit point of the bile duct from the liver and just before the point of entry into the duodenum) to effect a complete...
bile shunt, which permitted normal bile flow through the cannula when left intact. The bile duct and jugular vein cannulas were exteriorized directly behind the back of the head to facilitate installation of the dual swivel cannula apparatus. Animals were housed in an environmentally controlled room at 22°–24°C, with a relative humidity of 40–60% and a 12-hr light/dark cycle. Purina rodent chow and water were available ad libitum. Rats were fasted for at least 12 hr before dosing, with food withheld until at least 2 hr postdosing. Doses of TRI were administered in 2% polyoxyethylene-sorbitan monooate (Tween 80, Sigma Chemical Co.). TCEOH doses were administered in double distilled water. All doses were administered between 9:00 and 11:00 a.m. to coincide with the animal’s circadian rhythm and minimize stress. Upon dosing, animals were housed in Nalgene metabolism cages for the collection of urine and feces, in addition to the samples collected from the respective cannulas. Each metabolism cage was equipped with a dual swivel cannula apparatus to allow animals freedom of movement without damaging cannulas during the course of the study.

Experiments with Interrupted Bile Flow. Bile duct and jugular vein-cannulated F-344 rats were dosed in separate experimental sets (N = 4 each set) with 5, 20, and 100 mg/kg TCEOH and 100 mg/kg TCA doses by intravenous injection. Blood, bile, urine, and feces samples were collected as described. An experiment was conducted to determine the extent of bile secretion of metabolites from an i.d. dose of TRI (100 mg/kg). Bile duct and jugular vein-cannulated F-344 rats (N = 2) were dosed with radiolabeled TRI ([14C]TRI, 500 mg/kg) by injection into the bile duct return cannula. Bile samples were collected over a period of 24 hr. All bile samples were collected on ice, immediately weighed, and the volume determined via a pipet at the end of each time point. Bile samples were then placed in a freezer (−70°C) for storage awaiting analysis. TCA and TCEOH were found to be stable in urine for at least 24 hr (10); thus, no degradation of samples was expected. Urine and feces were collected over intervals of 0–8, 8–24, and 24–48 hr. All samples were analyzed in accordance with the analytical methods described in the next section.

Analytical Methods. Blood, bile, urine, and feces samples were prepared and analyzed for free and total TCEOH (i.e. total TCEOH is free TCEOH + conjugated TCEOH) and TCA in accordance with procedures established in our laboratory. Briefly, a 100 μl sample, 100 μl of internal standard (DPCA preparation), and 100 μl of saline (60%/ethanol 40%) are mixed in 2 ml reaction vials and sealed with rubber septum and aluminum crimp tops. All samples were split equally, with one-half analyzed for free TCEOH and TCA, and the other half analyzed for total TCEOH and TCA. β-Glucuronidase was added to the half being analyzed for total TCEOH, to hydrolyze the glucuronide to TCEOH, and both sample fractions and standards were incubated for 18 hr at 37°C. After β-glucuronidase incubation, 50% H2SO4 was added to the split samples for total metabolite analysis and the samples incubated for 2 hr at 80°C to hydrolyze any other conjugates (e.g. TCA conjugates). All samples were then extracted with ether and analyzed on a Hewlett-Packard Gas Chromatograph. TCA was detected as the ethyl ester after derivatization with diazothane. Results are presented as the mean values of at least four rats, with the error expressed as the standard error of the mean.

Bile samples from radiolabel studies were analyzed by first taking an aliquot, which was used to determine total radioactivity. The remainder of the sample was then incubated with β-glucuronidase (1250 units/sample) for 18 hr, followed by acid hydrolysis and heating at 80°C to ensure complete hydrolysis. Samples were then subjected to HPLC to separate metabolites. The HPLC used a Phenomenex C18 column and 1.0 ml/min flow rate, with methanol as the elution solvent. The HPLC column effluent was collected at predetermined time intervals (i.e. 0.5-min intervals) and the amount of radioactivity in each fraction determined by liquid scintillation counting. The liquid scintillation count data from the samples with β-glucuronidase, acid hydrolysis, and heating (i.e. hydrolysis treatment) provide an estimate of the amount of radioactivity that copurifies with each metabolite found in the bile.

Gas Chromatography. Analyses were performed on a Hewlett-Packard 5840A GC equipped with an electron capture detector (Hewlett-Packard model 18803B) and a Hewlett-Packard 7672A Automatic Liquid Sampler. The GC was equipped with a DB-5 (5% diphenyl and 95% dimethylpolysiloxane; 30 m × 0.32 mm i.d., 0.25 μm film thickness; J & W Scientific Co., Folsom, CA) fused silica (megabore) capillary column. Helium was used as the carrier gas at 5 ml/min. The GC was operated in the splitless mode. The postcolumn precpector sweep and detector make-up gas consisted of 95% argon/5% methane at a flow of 30 ml/min. An inlet temperature of 250°C, a column temperature of 90°C, and a detector temperature of 300°C were used in the analysis. Toward the end of the study, the Hewlett-Packard 5840A GC was replaced with a Hewlett-Packard 5890 GC because of equipment failure. The Hewlett-Packard 5890 GC was equipped with an electron capture detector and an automatic liquid sampler. It was set up with a DB-WAX (30 m; 0.53 mm Megabore; 1.0 μm film thickness; J & W Scientific) column. Helium was used as the carrier gas at 5 ml/min. The Hewlett-Packard 5890 GC was operated in split mode (3:1 split). Its postcolumn pre-detector sweep and detector make-up...
gas consisted of 95% argon/5% methane at a flow of 30 ml/min. An inlet temperature of 225°C, a column temperature of 90°C, and a detector temperature of 300°C were used.

**Pharmacokinetic Analysis.** $K_{\text{elim}}$'s were calculated using the equation:

$$K_{\text{elim}} = \frac{\ln C_1 - \ln C_2}{(T_2 - T_1)}.$$  

Formation rate constants were calculated from the extrapolation formation slope determined by the method of residuals. AUCs were calculated by the trapezoid method. Half-life ($t_{1/2}$) values were calculated using the equation:

$$t_{1/2} = \frac{0.693}{K_{\text{elim}}}.$$

**Statistical Analysis.** Comparisons of blood and urine mean values for with vs. without intact enterohepatic recirculation and the analysis of blood to bile concentration gradients were conducted using Student’s one-tailed $t$ test. Statistical significance was determined at a level of $p < 0.05$.

**Results**

Figure 2a shows TCA concentrations in systemic blood from experiments in which intravenous doses of TCEOH (100 mg/kg) were administered to F-344 rats ($N = 4$) with and without intact enterohepatic recirculation. Figure 2b shows TCEOH concentrations in systemic blood from experiments where intravenous doses of TCEOH (100 mg/kg) were administered to F-344 rats ($N = 4$) with and without intact enterohepatic recirculation.

If TCA and TCEOH, without enterohepatic recirculation, concentrations at each time point of fig. 2 (a and b) are subtracted from the corresponding with enterohepatic recirculation time points, the result is difference curves reflective of the TCA and TCEOH blood concentrations attributable to enterohepatic recirculation. These difference curves are presented in fig. 2c. The contribution from enterohepatic recirculation to the amount (i.e. based on AUC values) of TCEOH in systemic blood is ~36%, whereas 76% of the amount of TCA in systemic blood depends on enterohepatic recirculation of metabolites. Table 1 presents several kinetic parameters calculated for total TCEOH (free + glucuronide) in blood and bile for the 5, 20, and 100 mg/kg doses of TCEOH in F-344 rats. Table 1 also presents several kinetic parameters calculated for TCA in blood and bile for the 5, 20, and 100 mg/kg dose of TCEOH in F-344 rats. As can be seen, changes in blood concentrations of total TCEOH and TCA are basically proportional with dose, and reasonably consistent half-lives were observed at the different doses levels.

Total TCEOH is found in bile at high concentrations after an intravenous dose of 100 mg/kg TCEOH (fig. 3a). In bile, total TCEOH consists primarily of TCEOH glucuronide, whereas, in systemic blood, it is primarily unconjugated (i.e. free TCEOH). In addition, TCA was also found in the bile of F-344 rats administered an intravenous dose of 100 mg/kg TCEOH; but, in this case, the concentrations were generally in the same range found in blood (fig. 3b). The appearance of TCA occurs much later than TCEOH. Moreover, the time course of TCA concentrations in bile reflects its half-life in blood. Some TCA is still found in bile, from a single dose of TCEOH, 24 hr after dosing.

A linear relationship of total TCEOH in bile was found with intravenous doses of 5, 20, and 100 mg/kg TCEOH. Figure 4 shows the AUC vs. dose curve for the three doses of TCEOH. As can be seen, the total TCEOH secreted into bile from intravenous doses of TCEOH increases linearly with doses up to 100 mg/kg.

To isolate and examine the potential for enterohepatic recirculation of TCA, itself, an experiment was conducted wherein TCA was administered intravenously (100 mg/kg) to F-344 rats with both bile duct and jugular vein cannulas ($N = 5$). Figure 5 shows the concentration over time found in systemic blood and bile from a 100 mg/kg dose of TCEOH, 24 hr after dosing.
intravenous dose of TCA. The TCA concentration has already reached a maximum in bile at 1 hr. A fairly rapid decline in TCA is seen over the next 6 hr. This is followed by the more characteristic longer half-life decline from 8 to 24 hr. These data reinforce the observation that the bile/blood concentration ratio is 1, if the time lapse for bile collection is taken into account.

For comparative purposes, blood concentrations of TCA, free TCEOH, and glucuronidated TCEOH from an intraduodenal dose of TRI (100 mg/kg) were also followed (fig. 6). This experiment was conducted using jugular vein and bile duct-cannulated animals, wherein enterohepatic recirculation was left intact (i.e., animals with bile cannula shunts connected). Blood concentrations of TCA agree well with those found by Templin et al. (11). The TCA concentration reaches a maximum ~10 hr and then is eliminated gradually.

The concentrations of glucuronidated TCEOH found in bile after an intraduodenal dose of TRI (100 mg/kg) are much greater than free TCEOH concentrations (fig. 7). This relative relationship between glucuronidated and free TCEOH is reversed in systemic blood. Peak concentrations of TCEOH glucuronide in bile, after intraduodenal doses of TRI, are ~200 times higher than peak concentrations of TCEOH glucuronide in systemic blood.

Table 2 shows the relative recovery of total TCEOH and TCA in bile from a high radiolabeled dose of [14C]TRI (500 mg/kg). The values are presented as percentage of radioactivity recovered in the bile sample. As can be seen, most of the recovered 14C is associated with total TCEOH, and virtually all (95%) of the label is found in total TCEOH and TCA.

A significant concentration gradient for TCEOH, which is primarily TCEOH glucuronide in bile, can be seen between systemic blood and bile by examining figs. 2b and 3a. Concentrations are statistically different by Student’s one-tailed t test (p < 0.05). There is >5 times more total TCEOH in bile than in systemic blood at peak concentrations. The gradient is even greater (i.e., 200 times) for TCEOH glucuronide, because most of the TCEOH in bile is in the glucuronidated form (fig. 7), whereas only 33% (approximately) of the TCEOH in systemic blood is in the glucuronidated form (fig. 6).

Glucuronidated TCEOH and TCA in systemic blood are ultimately eliminated via the urine. Table 3 shows the amount of metabolites found in the urine after an intravenous dose of TCEOH. Values obtained from animals with intact enterohepatic recirculation path-

### TABLE 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Dose (mg/kg)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</th>
<th>AUC (µg·hr/ml)</th>
<th>K&lt;sub&gt;elim&lt;/sub&gt; (hr)</th>
<th>Half-life (hr)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</th>
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<tr>
<td>TCEOH</td>
<td>5</td>
<td>—</td>
<td>13.8</td>
<td>2.16</td>
<td>0.3</td>
<td>47.5 ± 12.8</td>
<td>62.6</td>
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<tr>
<td>TCEOH</td>
<td>20</td>
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<td>46.9</td>
<td>1.89</td>
<td>0.4</td>
<td>113.7 ± 27.0</td>
<td>204.8</td>
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<tr>
<td>TCEOH</td>
<td>100</td>
<td>—</td>
<td>287.5</td>
<td>1.02</td>
<td>0.7</td>
<td>411.3 ± 129.7</td>
<td>983.6</td>
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<tr>
<td>TCA</td>
<td>5</td>
<td>0.16 ± 0.07</td>
<td>1.6</td>
<td>0.09</td>
<td>7.7</td>
<td>0.5 ± 0.1</td>
<td>5.4</td>
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<tr>
<td>TCA</td>
<td>20</td>
<td>0.4 ± 0.04</td>
<td>12.1</td>
<td>0.06</td>
<td>11.6</td>
<td>0.5 ± 0.07</td>
<td>12.8</td>
</tr>
<tr>
<td>TCA</td>
<td>100</td>
<td>2.4 ± 0.5</td>
<td>38.3</td>
<td>0.07</td>
<td>9.9</td>
<td>2.5 ± 0.9</td>
<td>28.3</td>
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<tr>
<td>TCEOH</td>
<td>100</td>
<td>—</td>
<td>447.8</td>
<td>0.34</td>
<td>2.0</td>
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<tr>
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<td>160.4</td>
<td>0.06</td>
<td>11.6</td>
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C<sub>max</sub>, maximum concentration; EHC, enterohepatic recirculation.
ways were compared with those from animals without enterohepatic recirculation. It is important to note that the elimination of TCA, via the urine, is statistically different between animals with vs. without enterohepatic recirculation by Student’s one-tailed $t$ test ($p < 0.05$). The elimination of TCA, via the urine, is decreased by $\sim 80\%$ when enterohepatic recirculation is interrupted.

Only trace amounts of any of the Tri metabolites were found in fecal samples collected and analyzed from each of the experiments discussed previously.

**Discussion**

The concentrations of metabolites and their disappearance obtained in the experiments described herein are consistent with the magnitude and pattern reported by Templin et al. (9, 11) and Larson and Bull (12) after TRI administration. The blood concentrations of TCA agree well with those of Templin et al. (11), wherein the animals were dosed in a comparable manner. As previously reported, TCA concentrations peak $\sim 10$ hr, well after TRI effectively disappears from the blood, and then are gradually eliminated. The increased concentration in the free TCEOH follows an intermediate time course.

The delayed TCA production after oral doses of TRI seems to be largely accounted for by enterohepatic recirculation of TCEOH glucuronide. The postulated hypothesis is supported by the following findings: 1) the concentrations of free TCEOH occur with a time course that is consistent with its being an intermediate in the continued production of TCA; 2) TCEOH is the primary metabolite of TRI found in bile (i.e. 72% of the radioactivity from a radiolabeled dose of TRI was found as TCEOH in bile); 3) a significant concentration gradient of TCEOH glucuronide is established between blood and bile; 4) when TCEOH is administered to animals with interrupted bile flow, there is a significant decrease in both the maximum concentration and AUC for TCA in blood; and 5) urinary excretion of TCA is significantly decreased by interrupting the enterohepatic recirculation.

The likely pathway for this interaction is as follows: after an oral dose of TRI, it is rapidly converted to TCEOH. This reaction is probably favored over TCA formation as the result of reducing conditions found in the liver in vivo. Most of the TCEOH found is rapidly glucuronidated by glucuronyltransferase. A large fraction of the glucuronidated TCEOH is actively secreted into the bile, and a much smaller fraction is released into systemic blood by diffusion.
The glucuronidated TCEOH is transported into the bile and is available for enterohepatic recirculation. For this to happen, the glucuronidated TCEOH needs to be hydrolyzed by \( \beta \)-glucuronidase in the gut to release TCEOH, which can then be reabsorbed into systemic blood, where it will be reintroduced to metabolism to TCA in the liver or be reconjugated and recycled back through the biliary system.

The generality of this model is supported by prior studies in other species. Hobara et al. (13) show that TCA is produced in dogs from a TCEOH dose. In his study, Hobara administered free TCEOH (25 mg/kg) intravenously to both bile-cannulated and control dogs, with a resulting TCA concentration of 6.8 \( \text{mmol/ml} \) seen in the control animals after a period of 2 hr. No TCA was detectable in the blood of bile duct-cannulated dogs, reinforcing the quantitative importance of enterohepatic recirculation of TCEOH in the formation of TCA.

The linearity of dose relative to the formation of total TCEOH in bile from TCEOH intravenous doses of 5, 20, and 100 mg/kg was evaluated in this study. As was shown, the amount of total TCEOH produced from intravenous doses of TCEOH increases linearly with doses up to a dose of 100 mg/kg. Thus, data confirm the assumption of perfusion rate-limited kinetics for the conjugation and transport of TCEOH into bile for doses of TCEOH up to 100 mg/kg.

The addition of this enterohepatic recirculation pathway to a PBPK model for TRI is an important refinement, because it allows for more accurate prediction of the effective dose of TCA in the tissues of effect. A new PBPK model for TRI, which includes the enterohepatic recirculation pathway, was developed using the aforementioned experimental data. The development of this new PBPK model for TRI is described in a separate publication.

### References


