Short Communication

PLASMA BINDING OF TRICHLOROACETIC ACID IN MICE, RATS, AND HUMANS UNDER CANCER BIOASSAY AND ENVIRONMENTAL EXPOSURE CONDITIONS

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ABSTRACT:
Trichloroacetic acid (TCA), a mouse liver carcinogen, is a drinking water contaminant and a metabolite of solvents such as trichloroethylene and perchloroethylene. Because acidic drugs are often bound more strongly to human than to rodent plasma proteins, a study was undertaken to determine whether this was the case for TCA and to clarify the mechanistic bases for species differences. Equilibrium dialysis was used to measure in vitro binding of a range of TCA concentrations to plasma of humans, rats, and mice. Plots of observed data for free versus bound TCA concentrations were compared with simulations from each of three binding models: a single saturable site model; a saturable plus nonsaturable site model; and a two-saturable site model. Dissociation values ($K_d$) did not differ significantly from one species to another, but $N$ (number of binding sites/molecule) ranged from 2.97 for humans to 0.17 for mice. Binding capacities ($B_{max}$) for humans, rats, and mice were 709, 283, and 29 μM, respectively. The greater plasma protein binding of TCA in humans would be expected to not only increase the residence time of the compound in the bloodstream, but to substantially reduce the proportion of TCA that is available for uptake by the liver and other tissues. Species differences in the bound fraction diminished at very low, environmentally relevant TCA concentrations, but the percentage bound increased markedly. These findings suggest that the practice of using total blood levels of TCA as a dose metric in interspecies extrapolation of cancer risks needs to be re-examined.
target (hepatocyte) and the length of time it is present are major determinants of the magnitude of carcinogenic action. Species differences in plasma protein binding of a highly bound compound should be reflected in the concentration of unbound chemical in the plasma and target tissue. Relatively low plasma binding in rodents would result in relatively high exposure of their hepatocytes to TCA. The objective of the current work was to characterize and to compare the binding of TCA to mouse, rat, and human plasma in vitro. The data were analyzed to clarify the mechanistic basis of species differences in binding.

Materials and Methods

Chemicals. Trichloroacetic acid of 99.7% purity, phosphoric acid, and methanol were obtained from J.T. Baker (Phillipsburg, NJ). Sodium chloride, sodium phosphate (monobasic, anhydrous), potassium phosphate (dibasic, anhydrous), and n-hexane were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. Adult, male Sprague-Dawley (S-D) rats were supplied by Charles River Laboratories, Inc. (Wilmington, MA). The rats were maintained on a 12-h light/dark cycle at 21–23°C and 40 to 50% humidity in an AALAC-approved animal care facility. A diet of Laboratory Rodent Diet (PMI Feeds Inc., Richmond, IN) and tap water was provided ad libitum.

Plasma. Frozen, heparinized, pooled mouse and rat plasmas were obtained from Harlan Bioproducts for Science (Indianapolis, IN). Donor strain and sex were not specified. Blood was taken by cardiac puncture from the S-D rats to obtain fresh plasma. This plasma was stored at 4°C until use. Its binding performance was compared with thawed rat plasma at high [6.130 μM (1,000 μg/ml)] and low [0.61 μM (0.1 μg/ml)] TCA concentrations and found to be equivalent (data not shown). Pooled human plasma with EDTA as an anticoagulant was obtained frozen from Bioreclamation Inc. (Hicksville, NY). All plasma samples were filtered through 0.45-μm Millipore filters (Millipore Corporation, Bedford, MA) to remove precipitated fibrinogen. Total protein and albumin contents of plasma samples were measured by the biuret and the bromocresol green colorimetric methods, respectively.

Plasma Protein Binding Measurement. Binding of TCA to plasma constituents was determined by equilibrium dialysis, using a five-sample cell system from Spectrum Laboratories ( Rancho Dominguez, CA). Each cell was divided by a methyl ester cellulose dialysis membrane (45 mm, 12–14,000 mol. wt. cutoff) to produce two 1.3-ml reservoirs. One ml of plasma was injected into one reservoir. TCA in physiological buffer (100 mM sodium phosphate, 13 mM potassium phosphate, and 75 mM sodium chloride in triple-filtered deionized water, adjusted to pH 7.4) was injected into the other reservoir. Three replicates (n = 3) of 13 TCA concentrations were tested, ranging from 0.06 to 6.13 μM (0.01 to 1,000 μg/ml). The cells were sealed, submerged, and rotated in a 37°C water bath for 60 min. Pilot experiments revealed that TCA binding reached equilibrium by 60 min (data not shown). Plasma and buffer temperature and pH were maintained at 37°C and 7.4, respectively, to approximate the plasma binding environment in vivo. Mean analyte recoveries ± S.D. (n = 3) were 81 ± 18%, 99 ± 16%, and 82 ± 11% for human, rat, and mouse plasma, respectively.

At equilibrium, the concentration of TCA on the plasma side of the dialysis cells represented the total concentration (Ctotal), whereas that on the buffer side represented the free concentration (Cfree). The bound concentration could then be calculated as the difference between the total and free concentrations (Cbound = Ctotal − Cfree). A fluid shift of 10% of the original volume occurred in the direction of the buffer side of each cell. This behavior was compensated for by dividing the total concentration by 0.9 and the free side concentration by 1.1.

TCA Analysis. Aliquots were drawn from each side of the dialysis cell and processed for gas chromatographic (GC) analysis. The TCA in each sample was esterified with acidified methanol and extracted into n-hexane by the GC-electron capture procedure of Abbas and Fisher (1997). Chromatography conditions were as follows: HP-5 capillary column (0.25 mm × 30 m) with nitrogen as the carrier gas at a flow rate of 2.5 ml/min; inlet temperature = 260°C; detector temperature = 230°C; and oven temperature = 50°C. TCA standards were prepared daily and analyzed concurrently with the plasma- and buffer-side samples.

Fig. 1. Bound and free concentrations of TCA in human (open circle), rat (solid triangle), and mouse (open square) plasma in vitro for initial TCA concentrations (plasma side of equilibrium cells) ranging from 0.12 to 3.065 μM (A) and 0.12 to 6.13 M (B).

Data are expressed as mean concentrations (n = 3) ± S.D. Vertical error bars represent S.D. for the bound concentrations, and horizontal bars represent S.D. for the free.

Model Fitting. Plots of the bound fraction as a function of the free TCA concentration were fitted to different binding models to generate three parameter estimates: the number of binding sites per molecule of protein, N; the binding capacity, Bmax [N × P, the binding protein concentration (μM)]; and the dissociation constant KD (μM). Attempts were made to fit the data to expressions describing a single, saturable binding process (eq. 1), a saturable and a nonsaturable, linear binding process (eq. 2), and two independent, saturable binding processes (eq. 3).

\[
C_{\text{bound}} = \frac{(N \cdot P) \cdot C_{\text{free}}}{K_D + C_{\text{free}}} 
\]

(1)

\[
C_{\text{bound}} = A \cdot C_{\text{free}} + \frac{(N \cdot P) \cdot C_{\text{free}}}{K_D + C_{\text{free}}} 
\]

(2)

\[
C_{\text{bound}} = \left(\frac{N_1 \cdot P}{K_{D1}} + C_{\text{free}}\right) \frac{C_{\text{free}}}{K_{D1}} + \left(\frac{N_2 \cdot P}{K_{D2}} + C_{\text{free}}\right) \frac{C_{\text{free}}}{K_{D2}} 
\]

(3)

Fit of the data to the first model (eq. 1) was evaluated, since at least one saturable binding process was apparent (Fig. 1). The second model (eq. 2) was based on the hypothesis of Taira and Terada (1985), who proposed that serum albumin is capable of low-capacity (saturable, nonlinear), high-specificity as well as high-capacity, low-specificity (nonsaturable, linear) anion binding. The linear term of this model is governed by a unitless binding coefficient (A). This equation was used to fit TCA binding to rat plasma in vitro by Yu et al. (2000).
The fraction of TCA bound as a function of its nominal concentration is shown in Fig. 2. Human plasma exhibited the most pronounced binding over the entire range of concentrations. Binding was highest (86.8%) at the lowest testable TCA level (0.12 μM). The bound fraction remained relatively constant, with a mean of 81.6% across the concentration range. Estimates of the binding parameters for the saturable process at the lower end of the TCA concentration range. Attempts to fit the mouse and human data to the saturable plus nonsaturable model (eq. 2) were unsuccessful. The best fits were obtained for each species (Fig. 3) with the single saturable binding process model (eq. 1).

Attempts were made to fit the free and bound TCA concentration data to each of the three binding models to generate the best estimates of $B_{\text{max}}$, affinity, and $N$. The preferred model would be one that adequately described the saturable processes in the low and high TCA concentration ranges. Estimates of the binding parameters for the saturable site with eq. 1 were used as initial estimates for fitting data to the two-saturable process model (eq. 3). Unfortunately, a reasonable estimate of CV% and AIC values was not possible due to an inadequate number of data points to fully describe the apparent saturable process at the lower end of the TCA concentration range. Attempts to fit the mouse and human data to the saturable plus nonsaturable model (eq. 2) were unsuccessful. The best fits were obtained for each species (Fig. 3) with the single saturable binding process model (eq. 1).

Fitting was performed using total plasma protein and albumin concentrations. Much better fits were obtained for all species when albumin was used. For this reason, the albumin concentrations were used to define $P$. $P$ values for human, rat, and mouse plasma were 239, 190, and 196 μM, respectively. $N$ was found to be highest for human plasma (2.97), followed by rat (1.49) and mouse (0.17) plasma. $B_{\text{max}}$ was highest for the human and lowest for the mouse. Species differences in $K_d$ values were not biologically significant (Table 1).

### Discussion

The rank order of plasma protein binding (humans > rats > mice), that we found here for TCA, has been observed over the years for a substantial number of acidic drugs. Loscher (1978), for example, reported 94.8%, 63.4%, and 11.9% of valproate (di-n-propylacetate) to be bound to serum proteins of humans, rats, and mice, respectively. Despite numerous reports of the phenomenon, there have been few attempts to understand its basis. Cassidy et al. (1989) did identify two binding sites for flavone acetic acid in human plasma versus one in mouse plasma. Plasma protein binding and its role in the kinetics and toxicity of environmental contaminants have received even less attention.

In our study, some characteristics of the binding of TCA to plasma proteins differed from one species to another. The primary difference we observed was the number of binding sites per molecule of protein. Model fitting yielded $N$ values that ranged from 2.97 for humans to 0.17 for mice. The value of $<1$ suggested the presence in mouse plasma of other ligands competing for the TCA binding site. Dissociation constants did not differ significantly from one species to another, however, reflecting similar affinities. Lin (1989) likewise saw little difference in the affinities of human and rat plasma for diflunisal [5-(2',4'-difluorophenyl) salicylic acid], but found three distinct binding sites in human plasma and one in rat plasma.

In the current study, the relatively high binding capacity of human plasma for TCA was a product of its larger number of binding sites and its somewhat higher concentration of albumin. Plasma albumin concentrations are modestly higher in humans (Zhou et al., 2001). These investigators observed a reasonable degree of correlation between bound 5,6-dimethylxanthenone-4-acetic acid and the albumin content of plasma of humans, rats, rabbits, and mice. Fits of our TCA data to a saturable binding model were best when albumin rather than total protein was used. This observation is consistent with the concept that water-soluble, anionic compounds bind predominantly to albumin (du Souich et al., 1995).

The fraction of TCA bound to plasma proteins was both species- and TCA concentration-dependent. Our mouse value of 19%, however, is substantially lower than the 34...
to 55% measured by Templin et al. (1993). The reason for this disparity is unclear, although Templin et al. (1993) used Scatchard analysis over a narrower range of TCA concentrations to estimate binding parameters.

Species differences in plasma protein binding of TCA can significantly affect tissue disposition of the carcinogen. Although TCA is fully charged at physiological pH, it is apparently conveyed rapidly across cell membranes by a bidirectional monocarboxylate transporter (Poole and Halestrap, 1993). Jackson and Halestrap (1996) demonstrated efficient transport of chloroacetate and dichloroacetate by isolated rat hepatocytes. TCA is distributed uniformly outside the vasculature and does not bind appreciably to the liver or to any other tissue (Schultz et al., 1999). The concentration of TCA at the target site (i.e., hepatocyte) should therefore be largely governed by the concentration of free chemical in the plasma. The lowest chronic oral TCE dose to produce liver tumors in mice is 1,000 mg/kg (Bull, 2000). Peak blood TCA levels in B6C3F1 mice and in F344 rats gavaged with 1,200 mg of TCE/kg are reported to be ~580 and 300 μM, respectively (Abbas and Fisher, 1997; M. Lumpkin, S. Muralidhara, C. White, J. Fisher, C. Dallas, and J. Bruckner, manuscript submitted for publication). PBPK modeling by the latter researchers forecast peak blood TCA levels of 470 μM in humans who ingest 1,200 mg of TCE/kg. If these blood levels are equated to nominal TCA concentrations in our in vitro system, the free fraction of TCA in mouse plasma will be about 4-fold higher than in rat plasma and 5-fold higher than in human plasma. These results are consistent with the unique susceptibility of mice to TCA-induced liver tumors, and call into question the practice of using total blood levels of TCA in mice under bioassay conditions as dosimeters for prediction of human liver cancer risk.

It is apparent from data in the current report that the percentage of plasma TCA binding increases in each species as the TCA concentration decreases. At the lowest level we could test [0.12 μM (0.02 μg/ml)], ~87% binding occurred in human plasma. PBPK modeling predicted that an oral bolus dose of 0.02 mg of TCE/kg would produce a prolonged TCA C_{max} of 0.02 to 0.03 μg/ml, with only 0.005 to 0.006 μg of free TCA/ml in human blood (M. Lumpkin, S. Muralidhara, C. White, J. Fisher, C. Dallas, and J. Bruckner, manuscript submitted for publication). Likewise, a 70-kg adult who drinks 2 liters of water daily containing 700 parts per billion of TCE would receive 0.02 mg/kg of the chemical, assuming 100% absorption. Our work suggests that percentage binding of TCA may approach 100% at even lower levels of exposure. We observed convergence of the fraction bound to plasma of all three species at 0.12 μM TCA, suggesting binding to the same site. Future experiments could clarify the extent and species specificity of plasma binding of a range of trace levels of TCA. In light of the foregoing, the validity of using total blood levels of TCA as a dose metric when extrapolating from high-dose, animal bioassay data to predict human environmental cancer risks needs to be re-examined.

ATSDR (1997a) *Toxicological Profile for Tetrachloroethylene (Update)*. U.S. Agency for Toxic Substances and Disease Registry, Atlanta, GA.

ATSDR (1997b) *Toxicological Profile for Tetrachloroethylene*. U.S. Agency for Toxic Substances and Disease Registry, Atlanta, GA.


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