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INTERACTIONS OF CARBON TETRACHLORIDE AND PROMETHAZINE IN THE RAT—I

EFFECTS OF PROMETHAZINE ON THE CONCENTRATIONS OF CARBON TETRACHLORIDE IN BLOOD AND LIVER, AND ON THE PRODUCTION OF CHLOROFORM

COLIN J. REDDROP*†, WALTER RIESS† and TREVOR F. SLATER*

*Biochemistry Department, Brunel University, Uxbridge, UB8 3PH, Middlesex, U.K., and
†Pharmacological Chemistry Department, CIBA-GEIGY Limited, CH-4002 Basle, Switzerland

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Abstract—The effects of Promethazine (PM, 78 μmoles/kg body wt, i.p.) on the concentrations of CCl_4 in samples of blood and liver of male, fasted rats after oral dosing with CCl_4 have been determined. With an administered dose of CCl_4 of 13 moles/kg body wt the concentrations of CCl_4 in the blood and liver were measured using gas chromatography with a flame ionisation detector. It was found that Promethazine delayed absorption of CCl_4 from the gastro-intestinal tract by approximately 2 hr as judged by blood levels of CCl_4; the maximum blood concentration (C_{max}) and the total absorption of CCl_4 (assessed by the area under the plot of blood concentration vs time during the first 6 hr after administration of CCl_4) were not significantly changed by Promethazine treatment. Liver and blood measurements were carried out on each rat in this series and the ratio of the CCl_4-concentrations in liver:blood were found to lie within the range 8–12 when studied in the absence of Promethazine treatment.

Gas chromatography with electron capture was used on serial samples of blood from the same rat to measure CCl_4 and $CHCl_3$ concentrations following oral administration of 13, 6.5 or 1.3 mmoles CCl_4/kg body wt. The increase in blood CCl_4 levels at all doses of CCl_4 administered was delayed by about 2 hr by administration of Promethazine. The maximum blood concentrations of CCl_4 and total amount absorbed (judged by area under the plot of blood concentration vs time) were dose-related to the amount of CCl_4 administered with or without Promethazine administration. Blood concentrations of $CHCl_3$ were relatively constant over the range of CCl_4-doses used indicating that the metabolic production rate of $CHCl_3$ is saturated at rather low doses of CCl_4 administered.

Carbon tetrachloride is an extensively studied hepatotoxic agent (for reviews see [1, 2]); it has been used in widely ranging doses (0.05–5 ml/kg body wt) to produce liver injury in rats and other species. Information on the concentration of CCl_4 in liver with different doses is not extensive; reports in the literature concern either limited time points with one dose of CCl_4, or are semi-quantitative or even qualitative in nature. One objective of this study has been to provide data on the liver content of CCl_4 at various time points with different doses, and using methods of established analytical accuracy.

Carbon tetrachloride is known to be metabolised by the cytochrome P-450 system in liver endoplasmic reticulum [1–5], and the activated primary product is generally believed to be the trichloromethyl radical [6–9]. Direct evidence for the formation of this free radical under conditions *in vitro* and *in vivo* has recently been obtained by electron spin resonance spin trapping techniques [10, 11]. The trichloromethyl radical is electrophilic and readily forms chloroform by hydrogen abstraction from an appropriate donor; chloroform formation can be used, therefore, as one index of the formation of the trichloromethyl radical in biological environments. We have measured chloroform contents of rat liver (and blood) following the administration of various doses of CCl_4 in order to obtain information on the relationship between the tissue content of CCl_4 and chloroform production.

Some aspects of the hepatotoxicity of CCl_4 are diminished by the concomitant dosing of rats with Promethazine [12, 13]. This phenothiazine is an effective free radical scavenger *in vitro* [14] but its action *in vivo* on CCl_4-induced liver injury has been claimed to result largely from an indirect action on the absorption rate of CCl_4 from the gastro-intestinal tract [15, 16]. However, the latter studies concerned restricted time periods and were performed with high doses of CCl_4. Another objective of this study was to provide more extensive data on the liver contents of CCl_4 in the presence of Promethazine, and to investigate the effects of Promethazine *in vivo* on the production of chloroform.

Using precise analytical methods developed for these purposes [17, 18] we have studied also the relationship between the concentrations of CCl_4 and $CHCl_3$ in liver and blood using serial sampling procedures; the objective was to decide whether blood analysis provides data that are strictly proportional to liver concentrations.

MATERIALS AND METHODS

Chemicals. CCl_4 (for spectroscopy) and $CHCl_3$, diethyl ether and toluene (A.R.) from E. Merck, Darmstadt and propyl iodide (A.R.) from Fluka, AG, Switzerland were used throughout the experiments. Promethazine hydrochloride was a gift of SPECIA, Paris.

Analysis. CCl_4 and $CHCl_3$ were determined in samples of blood and liver by gas-liquid chromatography (GC) using flame ionization detector (FID) analysis of headspace vapours, or by the analysis of toluene extracts [18] using an electron capture detector (ECD).

Treatment of animals. Young, adult, male Tif RAI f (SPF) rats (CIBA-GEIGY), body wt approx. 200 g were fed on a standard diet (NAFAG 890 pellets) whilst maintained in a climate-controlled room with a 12 hr light/dark cycle. The rats were fasted for 15 hr in cages with stainless steel grids above the cage floor prior to experimental dosing. Tap water was available *ad libitum* throughout the fasting and post-administration periods.

The rats were weighed, etherized and administered a 13.0, 6.5 or 1.3 mmoles/kg dose of CCl_4 as a solution in light paraffin by intra-gastric cannula (i.g.) and, simultaneously, an intra-peritoneal (i.p.) injection of either PM 78 μmoles/kg (as a freshly prepared 1% (w/v) solution) or an equivalent volume of physiological saline. All doses were administed mid-morning (between 08.30 and 11.30) in volumes equivalent to 0.5 ml mixture/200 g body weight.

Sample collection. Liver homogenates and blood (at time of sacrifice): the rats were deeply etherized and the peritoneal and thoracic cavities were opened quickly. The inferior caval vein was severed cranially to the diaphragm and whole blood collected from the thoracic cavity in a heparinized syringe. Small glass sample tubes were well filled, snap-frozen on dry ice (−78°) and stored in a deep-freeze (−20°). The liver was excised, dried to remove excess blood, weighed in a tared flask and homogenized at 0° with a Bühler-HO sealed-chamber homogenizer using a strict homogenization procedure [18]. Well-filled sample tubes were then snap-frozen and stored as for blood, above. Head-space analysis using a FID [18] was conducted within 72 hr.

Blood by serial sampling: A heparinized micro-haematocrit tube was inserted into the retro-orbital capillary net of a lightly etherized rat. About 4 drops of blood emerging from the tube were collected in tared test-tubes containing a buffer extractant/internal standard mixture [18]. The tubes were then re-weighed to determine the exact sample weight extracted and submitted to immediate analysis using an EDC [18]. With care, blood samples may thus be collected in rapid succession over short periods (e.g. 10–15 min intervals for 30–60 min) and at longer intervals over extended periods (e.g. hourly 6–8 hr). These experiments were carried out in the laboratories of CIBA-GEIGY Limited, Basle.

RESULTS

CCl_4 *absorption and distribution*

The content of CCl_4 in 1 g samples of whole blood and liver homogenate obtained from the same rats

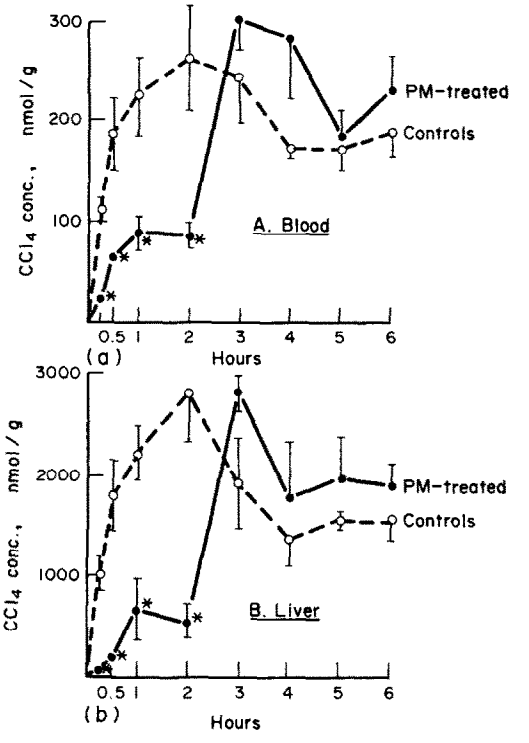

Fig. 1. Concentrations of CCl_4 (A) in rat blood and (B) in liver after administration of 133 mmoles CCl_4/kg body wt; one group (○) was concomitantly administered saline by intraperitoneal injection, and another group (●) was given Promethazine (78 μmoles/kg body wt). Mean values ± S.E.M. are shown; there were seven rats in each group. The asterisk (*) indicates $P < 0.05$ for the difference between the two groups at that particular time point. For other details see the text.

was determined by FID head-space analysis. Corrections were made for losses of CCl_4 that occur during collection, storage and handling of the samples [18]. The effects of the simultaneous administration of Promethazine on the absorption and distribution of CCl_4 in blood and liver are shown in Fig. 1. Absorption of CCl_4 is delayed by approx. 2 hr by the administration of Promethazine. Although not shown in Fig. 1, we measured the effects of Promethazine on the blood concentrations of CCl_4 and $CHCl_3$ at the additional time points of 7, 8, 9 and 24 hr post-dosing with CCl_4. There were progressive decreases in the concentrations over this time range; these additional data can be found in ref. 19.

Calculation of the liver:blood ratio of CCl_4 concentration for each individual rat produced the mean ±S.E.M. values shown in Fig. 2. The ratio for the saline-treated group fell between 8 and 12 over the time period studied; there was no statistically significant difference between these mean values in the saline-treated group. The overall mean ±S.E.M. for the saline-treated group was 9.3 ± 0.3. Promethazine treatment caused a marked decrease in the ratio during the first 2 hr of intoxication; this arises from a stronger effect of Promethazine on liver concentration compared to blood (Fig. 1). It

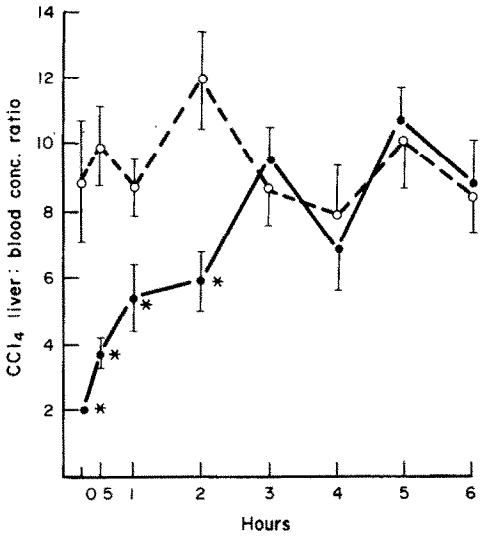

Fig. 2. The ratio of the concentrations of CCl_4 in liver : blood at various times after dosing with 13 mmoles CCl_4/kg body wt. The mean values ± S.E.M. shown were calculated from results used in Fig. 1. The asterisk (*) indicates $P < 0.05$ for the difference between the two groups at that particular time point. For other details see the text.

can be calculated from the data shown in Fig. 1 that the areas under the graphs in Fig. 1(A) and 1(B) over the time range 0–6 hr are not markedly different between the saline-treated and Promethazine-treated groups. The relevant data (in μmoles CCl_4/hr/g) are 1.2 and 1.1 for blood contents (saline and Promethazine groups respectively), and 11.6 and 9.0 for liver contents (saline and Promethazine groups respectively). In these experiments $CHCl_3$ was determined in blood and liver samples simultaneously with CCl_4. However, the amounts detected consistently bordered on the lower sensitivity limits of the FID head-space analysis method used [18]. Quantitative data assessment was therefore not possible but we were able to observe that the $CHCl_3$ concentration : time curves were parallel in blood and liver of both treatment groups, but at concentrations approx. 50–100 times lower than those of CCl_4. Accurate data relating to the production of $CHCl_3$ were obtained by the ECD technique described below.

CCl_4 and $CHCl_3$ concentrations in blood (ECD analysis)

Rats were administered different amounts of CCl_4 (1.3, 6.5 or 13 mmoles/kg body wt) by stomach tube, and either saline or Promethazine by intraperitoneal injection. Serial samples of blood were taken from each rat at five time points after administering the CCl_4; these samples were then analysed by the ECD method for CCl_4 and $CHCl_3$. The cumulative results are shown in Fig. 3.

Except for minor qualitative differences, the results obtained following FID head-space analysis were confirmed by ECD analysis. In each dose group, the principal effect of PM was to delay CCl_4 absorption by approx. 2 hr. C_{max} values for CCl_4 were obtained which corresponded to the ratio of the doses administered (10:5:1).

The graphs given in Fig. 3 show mean values obtained from the 4 or 5 separate plots obtained for each treatment group (see legends to Figs 3 and 4). The area under each graph for each individual rat for the time period 0–3 hr after administering CCl_4 was calculated and these values were used to construct Fig. 4. It can be seen that there is a linear relationship between the area under the curves for CCl_4 contents and the dose of CCl_4 administered. However, the areas under the curves for $CHCl_3$-contents show only a limited dose response relationship (Fig. 4(B)); this suggests that the metabolic conversion of CCl_4 to $CHCl_3$ is saturated at very low concentrations of CCl_4.

DISCUSSION

The results concerning the effects of PM on the absorption and distribution of CCl_4 during the first 3 hr of intoxication are generally consistent with those of both Nadeau and Marchand [16] and Castro *et al.* [20]. Using the same PM treatment as in this study, Nadeau and Marchand [16] reported a general lowering of the concentrations of CCl_4 in blood and liver; the differences were significant at 2 and 3 hr in the liver only. The study by Castro *et al.* [20] concluded that application of PM 30 min prior to CCl_4 administration has little effect on liver concentrations beyond +3 hr.

We have found a significant lowering of both blood and liver CCl_4 concentrations in the first 2 hr after PM administration. This appears to be a simple absorption delay as C_{max} and AUC values comparable with those of saline-treated controls are attained within the 6-hr period studied. Most histological assessments of the liver injury due to CCl_4 are made 18–24 hr post-dosing; it is evident from our data that for all but the first two hours of such a time period there is no marked diminution of CCl_4 concentration in the liver by Promethazine. This is consistent with the protective actions of Promethazine [12, 13] involving mechanisms additional to the simple absorption delay reported previously [15, 16]. In fact, the concentration of Promethazine in the liver during intoxication [17] is well above the level known to be effective in free radical scavenging reactions [14]. Since Promethazine also affects body temperature in the presence of CCl_4 [19] there are clearly several mechanisms by which Promethazine can attenuate the hepatotoxic action of CCl_4.

As many biochemical changes induced by CCl_4 intoxication are already apparent in the first few hours after administration [1, 2] and as the major effects of PM on the absorption and distribution of CCl_4 appear to be confined to the first 3 hr, we have based our discussion on an analysis of the concentration : time data for the 0–+3 period. An apparent saturation of CCl_4 metabolism via the $CHCl_3$ pathway at relatively low liver concentrations is suggested from the analysis of the dose : AUC relationship for CCl_4 and $CHCl_3$ in both control and PM-treated rats (Fig. 4).

Although the CCl_4 dose : AUC graphs take the standard linear form (Fig. 4(A)), the $CHCl_3$ data

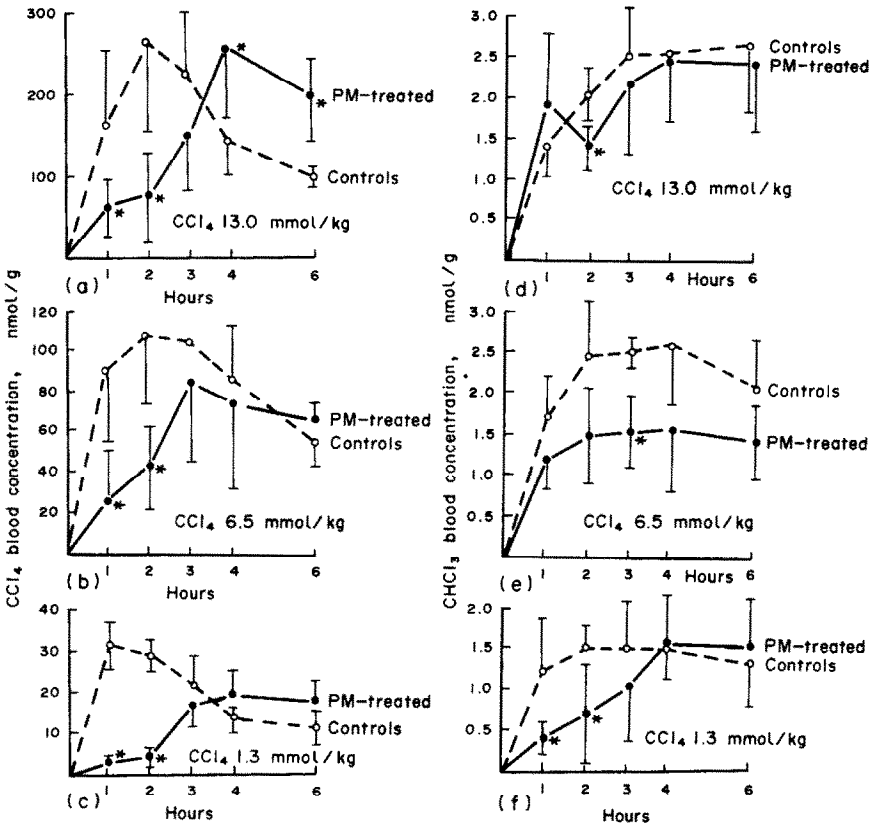

Fig. 3. Blood concentrations of CCl_4 and $CHCl_3$ at various times after dosing with CCl_4, and with different amounts of CCl_4. The estimations were performed on serial samples of blood from each rat in each group. Rats were divided into two groups (○, ●) as described in Fig. 1. The doses of CCl_4 administered were : 13 mmoles/kg body wt (graphs a and d); 6.5 mmoles/kg body wt (graphs b and e); 1.3 moles/kg body wt (graphs c and f). The concentrations of CCl_4 in blood are shown in graphs a–c, and of $CHCl_3$ in blood in graphs d–f. Mean values ± S.E.M. are given; the numbers of rats in each group were 4 graphs a, b, d, e, and 5 for c and f. The asterisk (*) indicates $P < 0.05$ for the difference between the two groups at that particular time point. For other details see the text.

analysis show a different profile (Fig. 4(B)). In control rats, $CHCl_3$ production (as measured by blood AUC) is relatively constant over a 10-fold CCl_4 dose range. However, in PM-treated rats, the low-dose group shows a significantly decreased $CHCL_3$ production.

The liver:blood concentration ratios for CCl_4 following a 13.0 mmoles/kg i.g. dose reached a relatively constant value (approx. 9:1) once absorption was complete (Fig. 2). Reasonably accurate estimates of CCl_4 liver concentrations may therefore be obtained from the measured CCl_4 blood concentrations. Assuming that the same liver:blood ratio applies to the other CCl_4 doses used in this study (a reasonable assumption, since all CCl_4 concentrations recorded here were dose-proportional), it is possible to estimate a CCl_4 liver concentration beyond which $CHCl_3$ production (as indicated by constant $CHCl_3$ blood levels) may be expected to be maximal: from Fig. 2(F) an approximately constant $CHCl_3$ blood concn is reach at 2–6 hr. The blood concn of CCl_4 over this time range is between 10 and 30 nmoles/g. Assuming the liver:blood ratio data described above also apply at very low doses, then the concentration of CCl_4 in liver between 2 and 6 hr is 100–300 nmoles/g. This estimation therefore predicts maximum $CHCl_3$ blood levels in the presence of CCl_4 blood and liver concentrations beyond approx. 30 and 300 nmoles/g respectively, i.e. concentrations only one-tenth of those pertaining for at least 6 hr after administration of CCl_4 13.0 mmoles/kg i.g., a dose used frequently in this model of hepatotoxicity.

In consequence, many studies on the metabolic activation and analyses of metabolites of CCl_4 under conditions *in vivo* have probably been made with doses of CCl_4 that have overwhelmingly saturated the metabolic activities of the pathways under study.

As demonstrated above and discussed previously [2], the dose of CCl_4 administered to rats is critical to all studies of this hepatotoxicity model, especially those which deal principally with aspects of metabolism. Using the existing GC methods and *in vivo* serial-sampling procedures, we hope that further clarification of the relationship between $CHCl_3$ blood concentrations and CCl_4 metabolism and the interation of PM with this relationship will be possible.

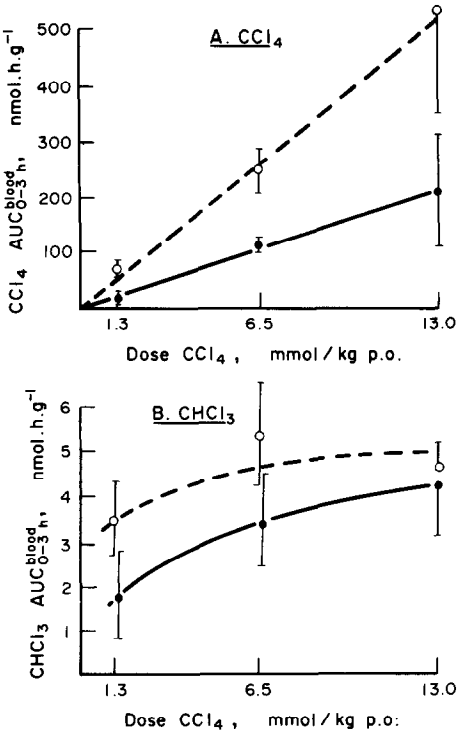

Fig. 4. Relationships between the contents of CCl_4 and $CHCl_3$ in blood during the period 0–3 hr after dosing and the amount of CCl_4 administered. The areas under the constituent curves used to construct Fig. 3 were calculated for each rat for the time range 0–3 hr after dosing; the mean values so obtained are shown plotted against the relevant dose of CCl_4 administered for the two groups (○, ●). Mean values ± S.E.M. are shown; the numbers of rats per group were as given in Fig. 3. The data are shown (A) for the blood concentrations of CCl_4 and (B) for $CHCl_3$. For other details see the text.

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