Urinary and Biliary Metabolic Patterns of Chlorothalonil in Germ-Free and Conventional Rats

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The metabolic fate of chlorothalonil, a broad spectrum fungicide that is known to be metabolized via glutathione conjugation, was examined through the analysis of urine and bile metabolites. The role of digestive microflora in the metabolism of chlorothalonil was assessed by comparing the metabolic patterns in germ-free and conventional rats. Low urinary and biliary excretion of radioactivity was observed in both conventional and germ-free rats. However, the urinary excretion of radioactivity was higher in conventional than in germ-free rats. Radio-HPLC analysis of urine and bile showed a complex metabolic profile in both conventional and germ-free rats. Methylthio metabolites of chlorothalonil were determined in ethyl acetate extracts of urine and bile of conventional and germ-free rats. These metabolites were excreted in a higher amount in the urine of conventional rats than in the urine of germ-free rats. This study shows the complexity of chlorothalonil metabolism and the role of the digestive microflora in chlorothalonil metabolism.

Keywords: *Chlorothalonil; metabolism; germ-free rats; glutathione conjugation; thiol metabolites*

INTRODUCTION

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile, CTL), a contact fungicide, is effective against a broad range of plant pathogens attacking many agronomic and vegetable crops.

In mammals, CTL is metabolized via glutathione conjugation and enzymatic cleavage to yield cysteine−glycine, cysteine, and *N*-acetylcysteine conjugates (Rosner et al., 1996) and thiolated compounds such as thiol (Killeen et al., 1990) and methylthio metabolites. Following chronic administration of CTL to rats, renal pathology may appear as hyperplasia and tumors (Wilkinson and Killeen, 1996). Over the past decade, evidence is accumulating that glutathione conjugation may be involved in a number of toxification reactions (Van Bladeren et al., 1988) such as activation by Cys β-lyases of cysteine conjugates to active thiol compounds. This was demonstrated for several halogenated alkenes as hexachlorobutadiene (Nash et al., 1984) and chlorotrifluoroethylene (Potter et al., 1981). The toxicity of chlorothalonil to rat kidney cells is attributed to the active thiol metabolites. Indeed, in vitro studies show that the di- and trithiol metabolites of chlorothalonil inhibit the kidney mitochondrial respiration (Wilkinson and Killeen, 1996). Moreover, it is speculated that the presence and/or the nature of the digestive microflora may play a significant role in the metabolic fate of chlorothalonil and in particular in the production of the thiolated compounds (Bessi et al., 1994). A metabolic study with chlorothalonil was conducted in germ-free rats (IPCS, 1996) and showed very low urinary levels, if any, for thiols. The thiols are speculated to be the nephrotoxic compounds responsible of the carcinogenicity of chlorothalonil. In contrast, studies conducted in conventional rats showed much higher levels of thiols in the urine (IPCS, 1996). This is in accordance with a previously published study (Hillenweck et al., 1997) in which we demonstrated in vitro the capability of the rat, dog, and human digestive microflora to biotransform chlorothalonil, chlorothalonil−diglutathione, and chlorothalonil−dicysteine to thiol metabolites. To verify if the results obtained in vitro can be confirmed in vivo, we undertook a comparative study in conventional and germ-free rats based on the analysis of the metabolites excreted in the urine and bile of both animal models.

MATERIALS AND METHODS

Chemicals. ^{14}C radiolabeled 2,4,5,6-tetrachloroisophthalonitrile (96.7% as determined by HPLC) and unlabeled CTL were provided by Ricerca Inc., Painesville, OH. The specific activity of chlorothalonil was 2.4 GBq/mmol.

2,5,6-Trichloro-4-thioisophthalonitrile, 2,5-dichloro-4,6-dithioisophthalonitrile, 5-chloro-2,4,6-trithioisophthalonitrile, and 5-chloro-2,4,6-trimethylthioisophthalonitrile (100% purity checked by TLC and HPLC) were provided by Ricerca Inc. 2,5,6-Trichloro-4-methylthioisophthalonitrile and 2,5-dichloro-4,6-dimethylthioisophthalonitrile were synthesized by diazomethane derivatization of the corresponding free thiolated standards as previously indicated (Hillenweck et al., 1997), and their structures were confirmed by GC/MS.

Animals, CTL Administration, and Collection of Samples. Four male Sprague−Dawley conventional (CV) rats, 8 weeks old, were received from Iffa-Credo (L'arbresle, France). The animals were given free access to food (A03, U.A.R. Villemoisson-sur-Orge, France) and water and were placed on study after an acclimation period of 7 days.

Four male Sprague−Dawley germ-free (GF) rats, 8 weeks old, were obtained from Iffa-Credo and maintained in a Trexler-type plastic isolator for an acclimation period of 7 days before the start of the experiment. Sterile food (A05-Ico-10,

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U.A.R.) and water were available ad libitum. Sterility of feces was checked by direct microscopic examination and by aerobic and anaerobic cultures on the day of arrival of the GF rats and before the start of the experiments. The GF rats were housed within the isolator in stainless steel metabolism cages that were designed to separate the urine from the feces.

An initial dose of 50 mg/kg chlorothalonil was given by gavage to rats as a homogeneous suspension of a mixture of labeled and unlabeled CTL in vegetable oil. Specific activities were 118.7 kBq/mg for CV rats and 111.7 kBq/mg for GF rats. The suspension administered to the GF rats was sterilized through a membrane filter (0.22 μm pore size, Millipore Corp.).

Urine was collected 12, 24, 48, and 96 h after dosing.

On the fifth day after the first administration, a second dose of CTL (50 mg/kg) was administered by oral intubation. Specific activities were 144.4 kBq/mg for CV rats and 116.2 kBq/mg for GF. Ninety minutes after dosing, rats were taken out from the isolator and anesthetized with an intraperitoneal injection of urethane dissolved in Ringers buffer (1.2 g/kg). A cannula was then implanted in the bile duct, and bile was collected on ice from 2 to 8 h after administration of the second radiolabeled dose. At the end of this period the rats were terminated by exsanguination. The total volume or weight of the excreta samples was measured, and aliquots of each sample were assayed for radiolabel content by liquid scintillation counting in a Packard Tricarb scintillation counter (model 4430, Packard Instrument Co., Downers Grove, IL) using Ultima Gold as the scintillation cocktail (Packard Instrument Co.).

Urine and Bile Analysis. Prior to HPLC analysis, bile samples were centrifuged (7000*g*, 2 h, 4 °C) on a Microsep microtube equipped with a 50K microfilter to eliminate the major part of bile proteins and in particular the enzymes involved in the biotransformation of glutathione conjugates. An aliquot was sampled before and after centrifugation for liquid scintillation counting. An aliquot of the filtrate was then acidified at pH 3 with 12.5 N HCl and extracted twice with ethyl acetate. HPLC analysis was performed on filtered bile samples and ethyl acetate extracts.

Crude urine was acidified at pH 3 with 12.5 N HCl and extracted twice with ethyl acetate. HPLC analysis was performed on filtered urine samples and ethyl acetate extracts.

HPLC Apparatus. Aliquots of urine and bile were analyzed by HPLC using a Spectra Physics (Les Ulis, France) system. The CTL metabolites were separated on a 250 × 4 mm C_{18}, 6 μm Ultrasep column (ICS, Lapeyrouse-Fossat, France) protected by a 10 × 4 mm C_{18} guard column (10 μm, ICS). Mobile phases were a mixture of 10 mM ammonium acetate, pH 3.5, and acetonitrile (98:2 for A and 2:98 for B) with a flow rate of 0.8 mL/min. A four-step HPLC gradient was used: linear gradient from 5 min (100% A) to 8 min (84% A) followed by 84% A for 15 min, linear gradient from 23 min (84% A) to 26 min (81% A) followed by 81% A for 15 min, linear gradient from 41 min (81% A) to 44 min (60% A) followed by 60% A for 15 min, and linear gradient from 59 min (60% A) to 63 min (100% B) followed by 100% B for 10 min. In these conditions, the retention time of CTL was 65.30 min. The eluent was monitored by UV detection with an LKB 2158 UV detector (Bromma, Sweden) equipped with a 254-nm UV filter and by ^{14}C detection with a Packard flo-one A500 using Flo-Scint II as liquid scintillation (Packard Instrument).

GC/MS Apparatus. A Nermag R.10.10.T mass spectrometer (Delsi Nermag Instruments, Argenteuil, France) was coupled to a Delsi DI 200 (Delsi Nermag Instruments) gas chromatograph fitted with a BPX5 25 m × 0.22 mm i.d. × 0.25 μm capillary column (SGE Ltd., Ringwood, Australia). The samples were injected in the splitless mode. Helium was used as the carrier gas at a flow rate of 1 mL/min and with a backpressure of 0.8 bar. The oven temperature was programmed as follows: 50 °C, 50 s; from 50 to 230 °C at 25 °C/min; and from 230 to 280 °C at 5 °C/min. The injector temperature was 270 °C and the interface temperature 270 °C. Mass spectra were generated at 70 eV with a source temperature of 220 °C.

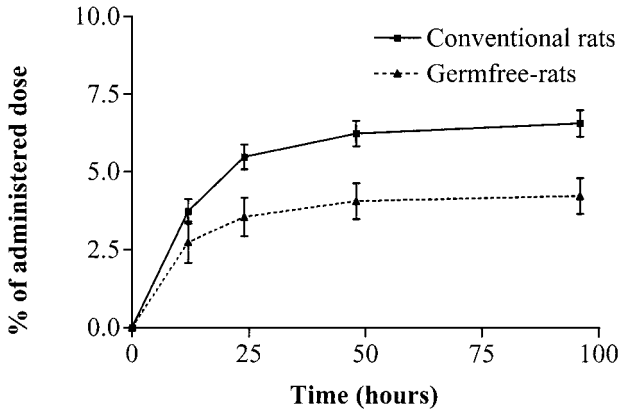

Figure 1. Cumulative excretion of radioactivity in urine of conventional (■) and germ-free (▲) rats.

Identification and Quantification of CTL and Thiolated Metabolites. The presence of CTL was assessed in the urine and bile of CV and GF rats, using selected ion monitoring (SIM) GC/MS detection. The mass detector was focused at *m/z* 264, 266, 229, and 194 for CTL monitoring ($[M]^{+\bullet}$, $[M + 2]^{+\bullet}$, $[M - 35]^{+\bullet}$, and $[M - 70]^{+\bullet}$, respectively).

The thiolated metabolites were selectively investigated in the urine and bile of CV and GF rats using the SIM GC/MS detection. Because they cannot be analyzed as such by GC/MS, detection was assessed by comparison of underivatized and methylated extracts. Ethyl acetate extracts were separated in two fractions, which were evaporated until dryness. In one case the residue was dissolved in dichloromethane and analyzed as such by GC/MS, whereas the other was derivatized using diazomethane (Hillenweck et al., 1997) just prior to GC/MS analysis as methylated compound. The mass detector was focused at *m/z* 276, 278, and 243 for monomethylthiotrichloroisophthalonitrile ($[M]^{+\bullet}$, $[M + 2]^{+\bullet}$, and $[M - 33]^{+\bullet}$, respectively), at *m/z* 288, 290, and 255 for dimethylthiodichloroisophthalonitrile ($[M]^{+\bullet}$, $[M + 2]^{+\bullet}$, and $[M - 33]^{+\bullet}$, respectively), and at *m/z* 300, 302, and 250 for trimethylthiochloroisophthalonitrile ($[M]^{+\bullet}$, $[M + 2]^{+\bullet}$, and $[M - 50]^{+\bullet}$, respectively). GC retention times of metabolites were compared with those of reference compounds.

The linear calibration curves of the methylthiolated compounds were constructed using increasing amounts (10−100 ng) of the appropriate methylthiolated standards. GC/MS peak areas were plotted against the amounts of standards injected, and the following linear calibration curves were obtained and used for the determination of methylthiolated metabolite amounts in the ethyl acetate extracts:

monomethylthiotrichloroisophthalonitrile:

$$y = 0.004495x;\ r^2 = 0.998$$

dimethylthiotrichloroisophthalonitrile:

$$y = 0.000159x;\ r^2 = 0.999$$

trimethylthiotrichloroisophthalonitrile:

$$y = 0.01524x;\ r^2 = 0.994$$

Statistics. Means were compared by the nonparametric Mann−Whitney test, and differences were considered to be significant when $p < 0.05$.

RESULTS AND DISCUSSION

Excretion of Radioactivity. In CV rats, cumulative totals of urine radioactivity accounted for 5.48 ± 0.79 and 6.56 ± 0.84% of the dose within 24 and 96 h, respectively, whereas in GF rats these percentages were 3.56 ± 1.22 and 4.23 ± 1.15 of the dose within 24 and 96 h, respectively (Figure 1). The major part of this radioactivity was eliminated during the first 24 h for

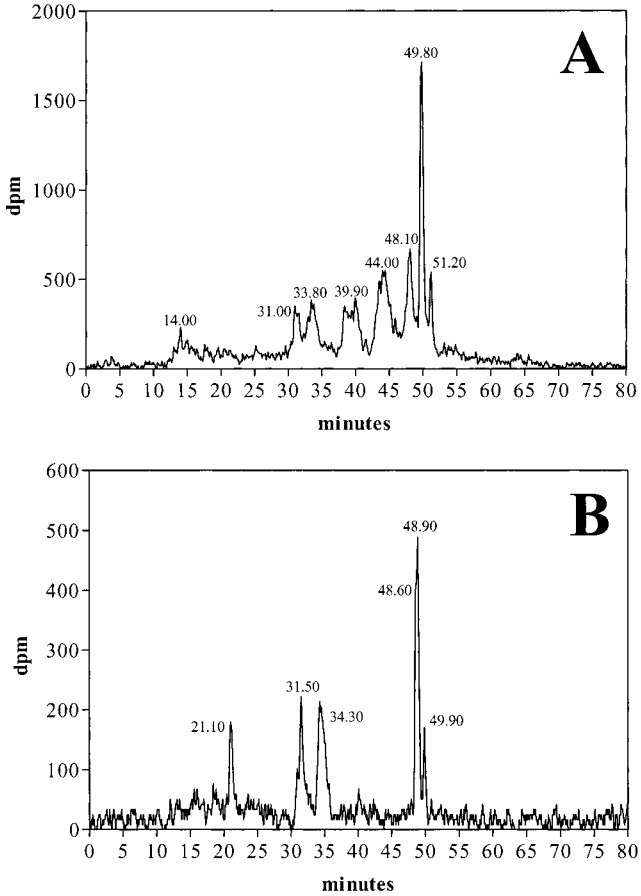

Figure 2. Radio-HPLC analysis of radioactivity excreted within 12 h in urine of CV (A) and GF (B) rats.

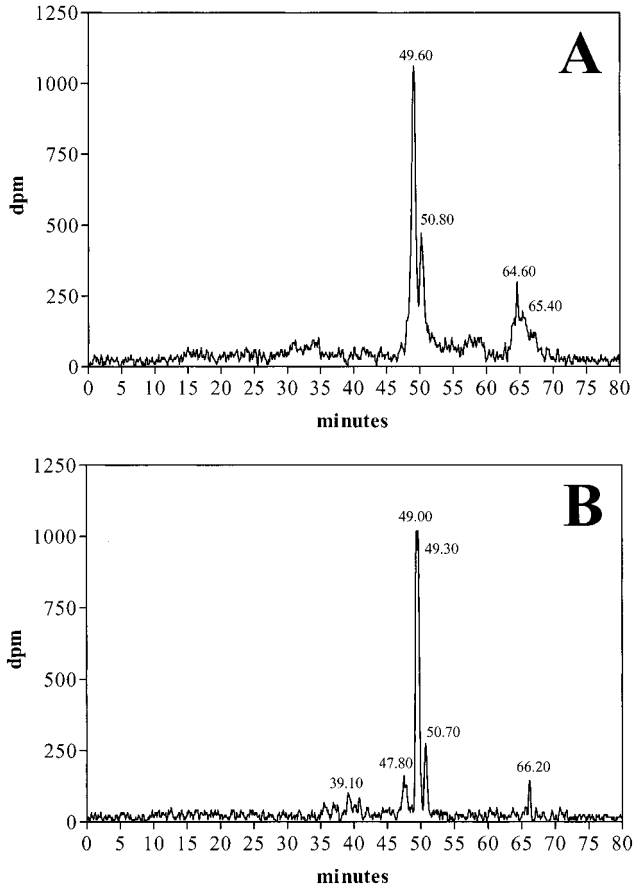

Figure 3. Radio-HPLC analysis of ethyl acetate extraction of radioactivity excreted within 12 h in urine of CV (A) and GF (B) rats.

both CV and GF rats. A statistical comparison of urinary excretion (96 h after dosing) between GF and CV rats showed that this elimination was significantly lower in GF rats ($p < 0.05$).

Bile was collected from three GF rats instead of four because the bile duct was damaged during the catheterization of one rat. Recovery of bile was performed from 2 to 8 h after the second oral administration of ^{14}C-CTL. During this period, the radioactivity recovered from bile accounted for 3.07 ± 0.71 and 1.77 ± 0.70% of the dose in CV and GF rats, respectively.

Low amounts of radioactivity were excreted in bile and urine of CV and GF rats. Rosner et al. (1996) report that, 72 h after an oral administration of 175 mg/kg of CTL to CV rats, the major part of the dose was recovered in feces as unchanged CTL. Moreover, we previously observed a low level of gastrointestinal absorption of CTL in everted gastrointestinal sacs of CV and GF rats (Hillenweck et al., 1998). This is in contrast with compounds having a similar structure, such as the herbicide dichlobenil (2,6-dichlorobenzonitrile). Indeed, dichlobenil is absorbed to a far greater extent than CTL: >70% of orally administered radioactivity was recovered from rat urine and bile (Bakke et al., 1988) for this compound.

Radio-HPLC Analysis. *Urine.* The crude urine collected from CV and GF rats was analyzed. Typical radio-HPLC profiles are presented in Figure 2 and show some differences between GF and CV rats. CV rats exhibited a major peak at 49.80 min and several minor peaks between 31 and 48.10 min, most of them being incompletely resolved. In GF rats, the same major peak (R_T of 48.90 min) was detected as well as more polar peaks between 20 and 35 min.

Radioactivity extracted with ethyl acetate from 0−12 and 12−24 h urine samples from CV rats accounted for 31.6 ± 7.4 and 42.6 ± 6.0% of the total radioactivity, respectively, whereas in GF rats these percentages accounted for 16.4 ± 2.3 and 18.3 ± 4.7%, respectively. These values suggest that the amount of polar metabolites (unextractable with ethyl acetate) was higher in GF rats than in CV rats.

The major urinary metabolite in the rat (R_T in the 49−50min range) was extractable with ethyl acetate as illustrated in Figure 3. The radio-HPLC profiles of the ethyl acetate extracts show minor peaks at 65−66 min, which correspond to the methylthiolated metabolites of CTL. These peaks appeared to be quantitatively more important in CV rats than in GF rats.

Bile. Bile from CV and GF rats was subjected to a 50 kDa filtration to eliminate the major part of proteins prior to radio-HPLC analysis. Radioactivity remaining in filtered bile was 90.5 ± 4.2 and 90.4 ± 5.9% of total biliary excreted radioactivity for CV and GF rats, respectively.

In each case, radiochromatograms exhibited a major peak or group of peaks at 48−50 min and a large number of poorly resolved compounds between 13 and 40 min (Figure 4).

Radioactivity extracted from the bile of CV rats with ethyl acetate was 15.4 ± 2.5% of the total radioactivity present in filtered bile, whereas in the GF rats this value accounted for only 4.9 ± 2.7% of the total radioactivity. As with urine, these values suggest that

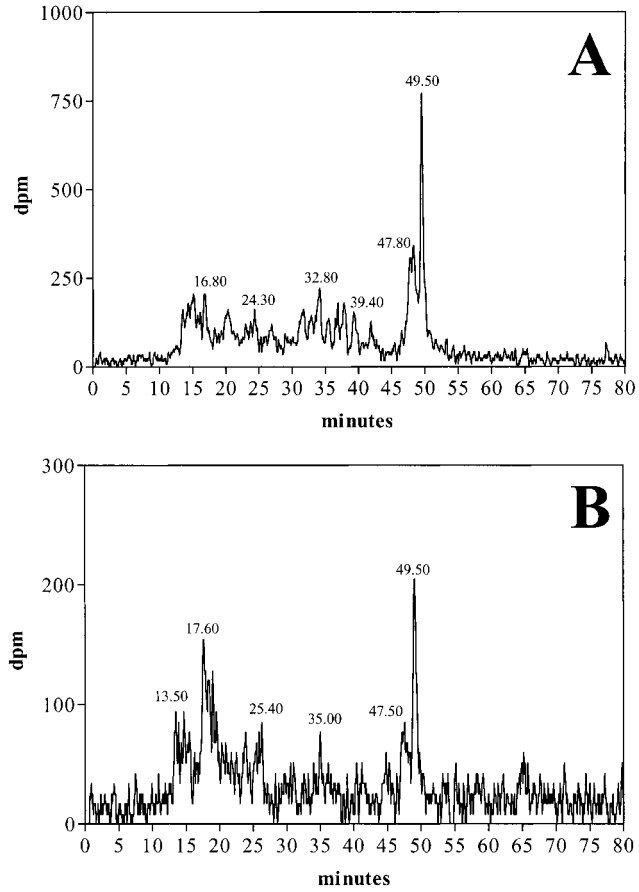

Figure 4. Radio-HPLC analysis of radioactivity excreted in bile of CV (A) and GF (B) rats.

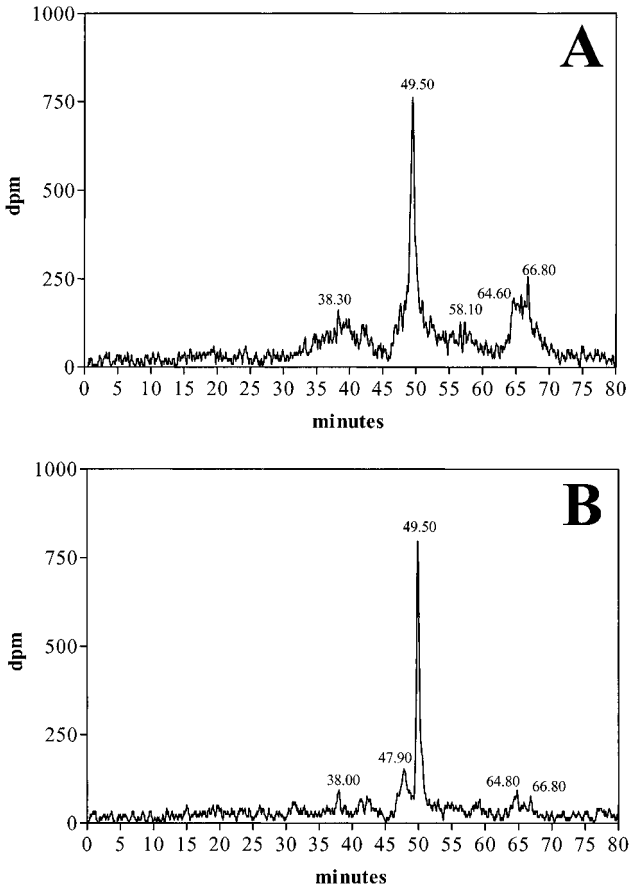

Figure 5. Radio-HPLC analysis of ethyl acetate extraction of radioactivity excreted in bile of CV (A) and GF (B) rats.

the amount of polar metabolites was higher in GF rats than in CV rats.

The major biliary metabolite (R_T = 49−50 min) was extractable with ethyl acetate (Figure 5). The radio-HPLC profiles of the ethyl acetate extracts show a group of unresolved peaks at 64−67 min, corresponding to the methylthiolated metabolites of CTL.

Urinary and biliary HPLC profiles from CV and GF rats show a complex in vivo metabolic pattern of CTL metabolites in these animals. A major part of the urinary and biliary radioactivity was unextractable with ethyl acetate, suggesting that the majority of metabolites are polar in nature.

The major compounds isolated from these excreta did not correspond to available standards and, despite several analytical approaches using various MS techniques, we failed in our attempts to identify them.

The presence of CTL in urine and bile of CV and GF rats was investigated by SIM GC/MS analysis. Unchanged CTL was unambiguously found in the bile sample of one CV rat (Figure 6) but was not identified from rat urine. This result is in agreement with our studies on the in vitro absorption of CTL in digestive everted sacs of rats in which we showed the passage of a small amount of unchanged CTL from the mucosal to the serosal side (Hillenweck et al., 1998). However, Rosner et al. (1996), using a diode array detector, failed to detect CTL from urine or bile samples of orally dosed rats. In our experiment, the amount of detected CTL in bile represented ∼1 ng. This was near the limit of GC/MS detection and may explain why Rosner et al., using UV detection during HPLC separation, did not identify CTL.

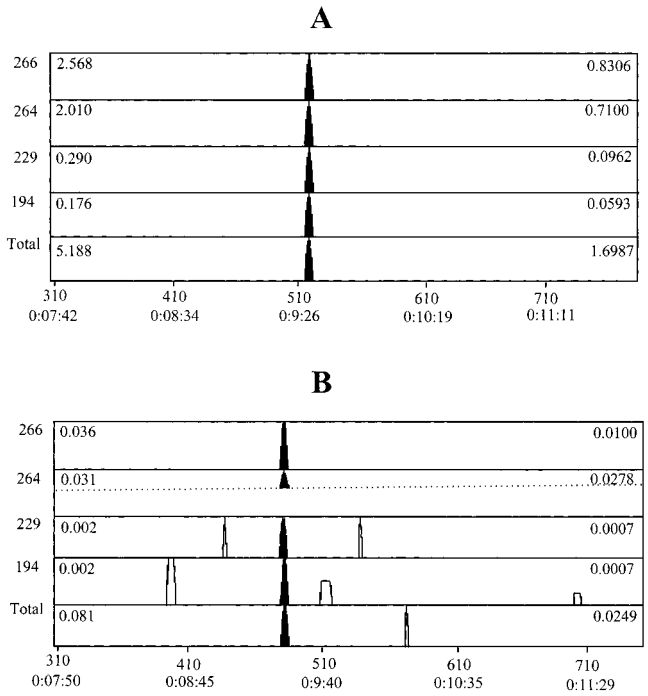

Figure 6. GC/MS analysis of CTL standard (A) and CTL excreted in CV rat bile (B).

Determination of Thiolated Metabolites. Thiol metabolites of CTL were determined by GC/MS analysis of ethyl acetate extracts from urine and bile obtained from CV and GF rats orally dosed with ^{14}C-CTL. The estimation was performed using the linear calibration curve for the mono-, di-, and trimethylthio metabolites

Table 1. Quantification of Thiol Metabolites of CTL in 0−12 h Urine and in Bile of CV and GF Rats[a]

	metabolite	CV rats	GF rats
urine	monomethylthio	<	<
	dimethylthio	$4.6 \times 10^{-2} \pm 2 \times 10^{-2}$*	$9.3 \times 10^{-3} \pm 2.6 \times 10^{-3}$
	trimethylthio	$4.2 \times 10^{-3} \pm 2.6 \times 10^{-3}$	$6.9 \times 10^{-4} \pm 9 \times 10^{-4}$
	total	$4.97 \times 10^{-2} \pm 2.1 \times 10^{-2}$	$10^{-2} \pm 2.2 \times 10^{-3}$
bile	monomethylthio	<	<
	dimethylthio	$3.9 \times 10^{-3} \pm 4 \times 10^{-3}$	$3.8 \times 10^{-3} \pm 3.3 \times 10^{-3}$
	dithiol	6.5×10^{-3}	<
	trimethylthio	$1.6 \times 10^{-3} \pm 1.4 \times 10^{-3}$	$1.3 \times 10^{-3} \pm 3.2 \times 10^{-3}$
	total	$9.4 \times 10^{-3} \pm 5.8 \times 10^{-3}$	$2.9 \times 10^{-3} \pm 1.5 \times 10^{-3}$

[a] Results are expressed as percent of administered radioactivity (mean ± SD, $n = 4$). <, under the detection threshold. *, significantly different at $p < 0.05$ between CV and GF rats.

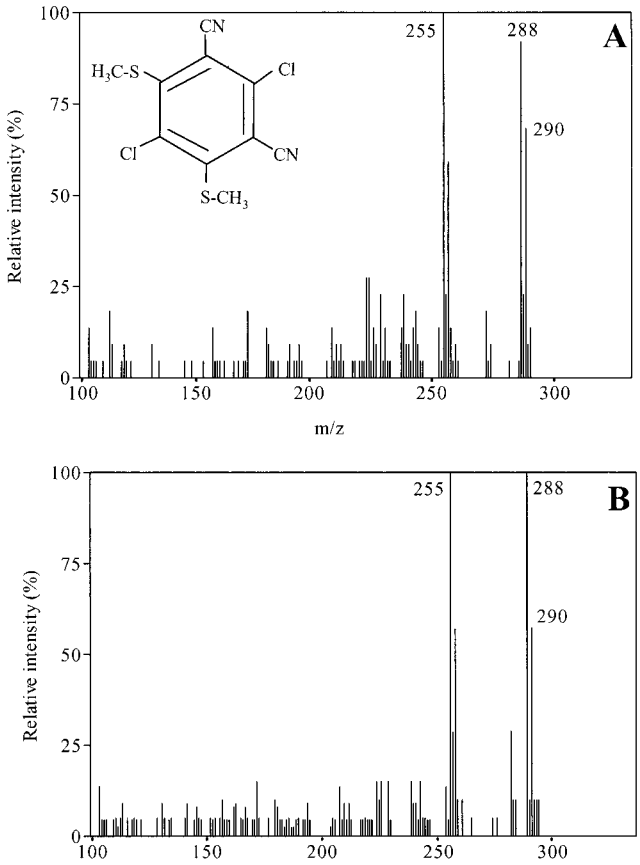

Figure 7. Mass spectra of 2,5-dichloro-4,6-dimethylthioisophthalonitrile standard (A) and metabolite recovered from CV rat urine (B) (GC/MS analysis).

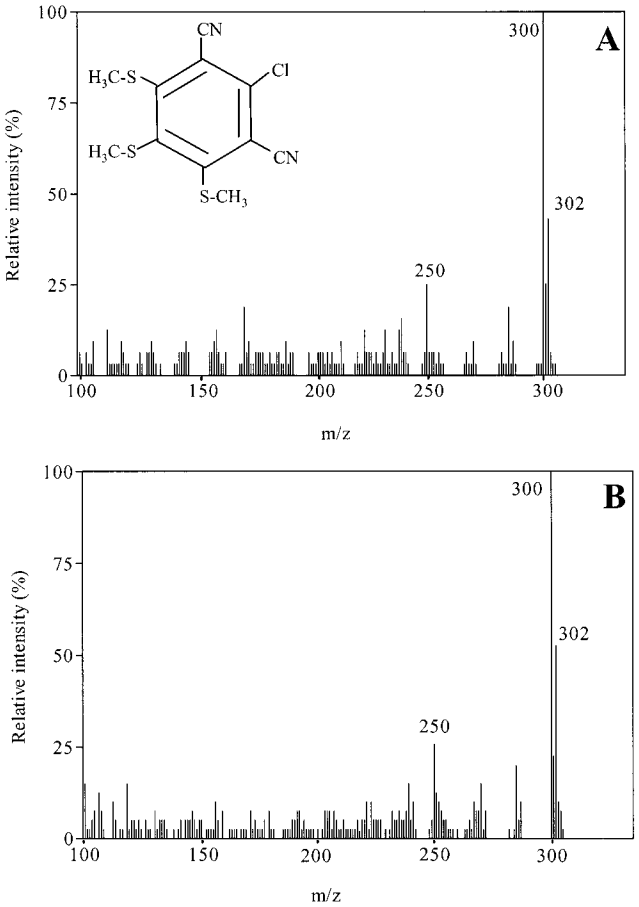

Figure 8. Mass spectra of 2-chloro-4,5,6-trimethylthioisophthalonitrile standard (A) and metabolite recovered from CV rat urine (B) (GC/MS analysis).

of CTL. Results are presented in Table 1 and are expressed as percent of the administered dose. The di- and trimethylthiolated metabolites of CTL were identified before and after derivatization in each case (Figures 7 and 8). The analyses demonstrate that in rat, thiolated metabolites were mainly excreted in urine and corresponded to dithiolated compounds. The amount of the dimethylthio metabolites in urine was significantly higher in CV than in GF rats ($p < 0.05$); however, no significant difference was observed in the urinary excretion of the trimethylthio metabolites between CV and GF rats. Thiolated compounds found in bile were predominantly metabolites having two or three thiol groups and presumably were degradation products of di- or triglutathione conjugates of CTL. The methylthio metabolites of CTL were detected in comparable amounts in the bile of CV and GF rats, but they were excreted in higher amount in urine than in bile of CV and GF rats ($p < 0.05$). This is in agreement with the tissue distribution of γ-glutamyl transpeptidases (γGT) and Cys-β lyases, which are the key enzymes for the formation of thiol compounds. Indeed, the kidney possesses one of the highest levels of γGT and Cys β-lyase activities (Commandeur, 1995). The total amount of thiolated metabolites excreted in the urine of the CV rats was significantly higher than the corresponding value in the GF rats ($p < 0.05$). However, this difference between GF and CV rats was less than that suggested by previous data (IPCS, 1996). Indeed, the IPCS report includes a review of studies in which, after a single oral dose of 50 mg/kg CTL, thiol derivatives of CTL were determined from urine of GF rats and were 50 times less than that obtained for CV rats. This difference from the current work may be related to the vehicles used:

in our study, the dose was administered in vegetable oil, whereas in the IPCS study, the dose was administered as an aqueous suspension. However, our results and those reported by IPCS (1996) show a difference in the amount of urinary thiolated metabolites between CV and GF rats, even if these metabolites were recovered in small quantities. In bile, the main difference between GF and CV rats was the presence of unmethylated dithiol compounds in GF animals (Table 1), suggesting that the digestive microflora were involved in the alkyaltion of thiols. This is in accordance with in vitro data showing the transformation of CTL in digestive contents of rat, dog, and human (Hillenweck et al., 1997).

Digestive microflora possess all of the enzymes involved in the mercapturic acid pathway (Bakke and Gustaffson, 1986) and especially in the production of thiol metabolites because a high Cys β-lyase activity has been detected in gastrointestinal bacteria (Larsen, 1985). The influence of digestive microflora in the metabolism of compounds that undergo glutathione conjugation was demonstrated for propachlor (Bakke et al., 1980), 2-acetamido-4-(chloromethyl)thiazole (Bakke et al., 1981), and paracetamol (Mikov, 1994). Bakke et al. (1980, 1981) showed that the digestive microflora are responsible for the formation of methylthiolated metabolites of propachlor and 2-acetamidothiazole, whereas GF rats quantitatively metabolize those latter compounds to mercapturic acid conjugates. The comparison in paracetamol metabolism between GF and CV mice performed by Mikov (1994) is consistent with an active role of the digestive flora in the formation of thiomethylparacetamol.

From the results of these experiments, we conclude that CTL is metabolized in rats through a complex metabolic pathway, and we confirm that the intestinal microflora are involved in this metabolism after oral administration of CTL in rats.

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