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# EFFECT OF HEXACHLOROPHENE, TRIBROMSALAN, TRICHLORCARBAN AND CLOFLUCARBAN ON HEXOBARBITAL SLEEPING TIME AND HEPATIC DRUG-METABOLIZING ENZYME ACTIVITY *IN VITRO* IN THE RAT*

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**Abstract**—Oral administration of the antibacterials hexachlorophene, tribromsalan and trichlorcarban at doses of 50, 10 and 40 mg/kg, respectively, caused significant increases in hexobarbital sleeping time in male Wistar rats, while an oral dose of 40 mg/kg of cloflucarban gave only a slight increase. The four halogenated antibacterials inhibited hepatic microsomal aminopyrine *N*-demethylase and aniline hydroxylase activities *in vitro* but had no effect on hepatic microsomal NADPH–cytochrome *c* reductase activity. The addition of each of the antibacterials to hepatic microsomes produced a type I difference spectrum. The spectral binding constants for interaction of hexachlorophene, tribromsalan, trichlorcarban and cloflucarban with hepatic microsomal cytochrome P450 were 33, 62, 66 and 40 $\mu$M respectively. The per cent of *in vitro* inhibition of hepatic microsomal mixed-function oxidase activity by these agents increased with increasing incubation times. The addition of bovine serum albumin to the incubation mixtures partially reversed the inhibition of microsomal drug metabolism caused by the halogenated antibacterial agents *in vitro*.

Halogenated antibacterial agents such as hexchlorophene [2,2'-methylenebis-(3,4,6-trichlorophenol); HCP] have been used in a variety of soaps, cosmetics and other consumer products. Because of serious toxic effects associated with the use of HCP [1], other halogenated antibacterial agents, notably the salicylanilides and carbanilides, have largely replaced HCP in personal care products.

One feature that the halogenated antibacterial agents appear to have in common is the ability to bind strongly to biological membranes and to disrupt membrane functions. For example, HCP and other bisphenols are potent uncouplers of oxidative phosphorylation [2, 3] and cause hemolysis of red blood cells *in vitro* [4]. Halogenated salicylanilides exhibit a similar hemolytic activity [5]. HCP has also been shown to bind strongly to rat liver microsomes *in vitro*, to inhibit hepatic microsomal mixed-function oxidase activity *in vitro*, and to prolong hexobarbital sleeping time in rats [6].

Since relatively little is known about the toxicity and biochemical effects of the newer halogenated antibacterial agents, the current studies were conducted to determine the effect of tribromsalan (3,4',5-tribromosalicylanilide; TBS), trichlorcarban (3,4,4'-trichlorocarbanilide; TCC) and cloflucarban (4,4'-dichloro-3-trifluoromethylcarbanilide; TFC) on drug-metabolizing enzymes in the rat. A preliminary account of this work has been published [7].

## MATERIALS AND METHODS

*Chemicals.* Horse heart cytochrome *c* (type VI), isocitrate dehydrogenase (type IV), D,L-isocitric acid (type I), NADPH and bovine serum albumin (BSA) were purchased from the Sigma Chemical Co. (St. Louis, MO). HCP was a gift of the Givaudan Corp. (Clifton, NJ), TCC was supplied by the Monsanto Co., TBS was purchased from K & K Laboratories, Inc. (Plainview, NY), and TFC was obtained from the Geigy Chemical Corp. (Greensboro, NC). The antibacterial agents were recrystallized from isopropyl alcohol–water prior to use. [Carboxy-$^{14}$C]TCC (sp. act. 1.37 $\mu$Ci/$\mu$mole) was a gift from New England Nuclear (Boston, MA). [Carbonyl-$^{14}$C]TBS (sp. act. 57 $\mu$Ci/$\mu$mole) was synthesized in this laboratory by a two-step procedure [8, 9]. Aquasol was purchased from New England Nuclear. Other chemicals were of the highest purity available commercially.

In vivo *studies.* HCP was administered orally in corn oil at a dose of 50 mg/kg, while TFC and TCC were administered orally at a dose of 40 mg/kg. TFC and TCC are less soluble in corn oil than HCP, which limited the dose of TCC and TFC (40 mg/kg) that could be administered conveniently in the corn oil vehicle. TBS was administered at a lower dose (10 mg/kg) since the majority of rats died after a 15 mg/kg oral dose of TBS.

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HCP has been shown to prolong hexobarbital sleeping time with the maximum effect occurring 6 hr after administration of the bisphenol [6], which corresponds to maximum concentrations of radioactivity in the plasma and in the liver after the oral administration of HCP*. Preliminary studies were conducted to determine maximum plasma levels of radioactivity after the oral administration of [$^{14}$C]TCC (36 mg/kg; sp. act. 0.125 $\mu$Ci/mg) and [$^{14}$C]TBS (10 mg/kg; sp. act. 0.19 $\mu$Ci/mg). Blood samples were obtained by incising the rat tail veins with a scalpel at 2 hr intervals. Blood plasma (0.1 ml) was added directly to 15 ml of Aquasol scintillation mixture, and the radioactivity was counted in a model 3375 Packard Tri-Carb liquid scintillation counter. Maximum concentrations of radioactivity appeared in the plasma 6 hr after the oral administration of TCC and TBS. It was assumed that the highest concentrations of TFC and its metabolites also occurred in the plasma approx 6 hr after oral administration since Hiles [10] has reported that the degree of absorption, rate of elimination and tissue distribution were almost identical for TCC and TFC when the same dosing method was used.

*Preparation of microsomes.* Livers were obtained from male Wistar rats (200–250 g), raised at Oregon State University, which had been fasted overnight prior to death. The rats were killed by decapitation and the livers were rapidly removed and washed with ice-cold 0.154 M KCl. Microsomes were prepared at 4° from livers which were homogenized in 4 vol. of 0.154 M KCl using a Potter–Elvehjem homogenizer and a motor-driven Teflon pestle. The homogenate was centrifuged at 9000 *g* for 20 min, and the resulting supernatant fraction was then centrifuged at 105,000 *g* for 30 min. The microsomal pellets were resuspended in 0.154 M KCl so that a 1-ml suspension contained 4 mg of microsomal protein. In experiments in which hepatic microsomes were used for spectral studies, the microsomal pellets were resuspended in one-half the original volume of 0.154 M KCl and recentrifuged at 105,000 *g* for 30 min. The pellets were resuspended in 0.154 M KCl containing 50 mM Tris–HCl, pH 7.4. Protein content was determined by the method of Lowry *et al.* [11] using BSA as the standard.

*Assays.* Spectral changes induced by the interaction of the halogenated antibacterials with hepatic microsomal cytochrome P450 were recorded with an Aminco DW2 split beam spectrophotometer as described by Estabrook *et al.* [12]. NADPH–cytochrome *c* reductase activity was assayed at 25°, as described by Baron *et al.* [13], by measuring the rate of reduction of cytochrome *c* by following the change in absorbance at 550 nm after the addition of NADPH to the sample cuvette at a final concentration of 90 $\mu$M. Both the sample and reference cuvettes contained 0.1 mg of microsomal protein, 36 $\mu$M cytochrome *c*, and 0.91 mM KCN in a 50 mM $KH_2PO_4$ buffer, pH 7.7 (2.2 ml volume). Varying concentrations of antibacterial agents were delivered in a fixed volume of acetone to the sample cuvette. The reference cuvette received an equal volume of acetone (25 $\mu$l). The millimolar extinction coefficient for the difference in absorbance between reduced and oxidized cytochrome *c* is 21 [14].

The methods of Schenkman *et al.* [15] were utilized to measure aminopyrine *N*-demethylase and aniline hydroxylase activities in rat hepatic microsomal suspensions. Incubation mixtures (2 ml) containing 4 mg of microsomal protein and an NADPH generating system were usually preincubated at 37° with varying concentrations of antibacterial agents (added in a fixed volume of 50 $\mu$l acetone) for 5 min prior to the addition of either 1 ml of 24 mM aminopyrine or 1 ml of 15 mM aniline. Control incubations received 50 $\mu$l acetone alone. Aminopyrine *N*-demethylase activity was estimated by measuring formaldehyde formation using the procedure of Nash [16], while aniline hydroxylase activity was determined by measuring *p*-aminophenol formation [15].

*Statistical analyses.* The $I_{50}$ values shown in Table 2 were calculated from the semilogarithmic plots of the respective data. Statistical analysis of the *in vivo* data was made by Student's *t*-test [17]. The spectral binding constant ($K_s$) was determined graphically by plotting the reciprocal of the changes in absorbance vs the reciprocal of various concentrations of antibacterials and calculating the reciprocal of the negative intercept with the abscissa [12].

## RESULTS

HCP, when administered orally to rats at a dose of 50 mg/kg in corn oil, was shown to alter hexobarbital sleeping time [6]. Three other antibacterial agents, TBS, TFC and TCC, were selected to compare their effects with HCP on drug-metabolizing enzyme activity *in vivo*. Pretreatment of rats with oral doses of antibacterials in corn oil resulted in prolonged hexobarbital sleeping time (Table 1). Statistically significant increases in sleeping times were observed in rats which had been given HCP, TBS and TCC, while only a slight increase in sleeping time was seen with TFC-treated rats.

The effects of the same antibacterial agents, HCP, TBS, TFC and TCC, on rat hepatic mixed-function

Table 1. Effect of hexachlorophene, tribromsalan, cloflucarban and trichlorcarban treatment on hexobarbital sleeping time in male Wistar rats

| Treatment | Dose (mg/kg) | Sleeping time* (min) | Percentage increase from control values |
|---|---|---|---|
| Corn oil | 0 | 52 ± 2 | 0 |
| HCP | 50 | 64 ± 5† | 23 |
| TBS | 10 | 62 ± 5† | 19 |
| TFC | 40 | 57 ± 6 | 10 |
| TCC | 40 | 73 ± 5† | 40 |

* Rats which had been fasted overnight were given hexobarbital intraperitoneally (100 mg/kg) 6 hr after the oral administration of a corn oil solution of the antibacterial agents. Each value represents the mean ± S. E. M. of hexobarbital sleeping times determined from at least three rats from three experiments.

† Denotes a significant difference from control values ($P < 0.05$) by one-tailed Student's *t*-test.

* D. R. Buhler, unpublished results.

Table 2. Inhibition of aminopyrine *N*-demethylase activity and aniline hydroxylase activity in the presence of hexachlorophene, tribromsalan, trichlorcarban and cloflucarban*

| Drug concn (nmoles/mg protein) | ($\mu$M) | Aminopyrine *N*-demethylase activity (nmoles HCHO/mg protein/20 min) | | | | Aniline hydroxylase activity (nmoles/mg protein/20 min) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | HCP | TBS | TCC | TFC | HCP | TBS | TCC | TFC |
| 0 | 0 | 78 ± 19 | 64 ± 6 | 66 ± 10 | 67 ± 3 | 5.8 ± 1.0 | 5.2 ± 0.6 | 6.0 ± 0.8 | 6.8 ± 0.6 |
| 19 | 25 | 59 ± 5 (24) | 52 ± 4 (19) | 55 ± 4 (16) | 57 ± 6 (15) | 5.2 ± 0.2 (10) | 4.0 ± 0.3 (24) | 5.5 ± 0.1 (8) | 5.6 ± 0.2 (17) |
| 38 | 50 | 44 ± 7 (43) | 39 ± 5 (39) | 49 ± 6 (25) | 48 ± 3 (29) | 4.2 ± 0.1 (27) | 3.8 ± 0.2 (26) | 4.4 ± 0.2 (26) | 5.0 ± 0.3 (27) |
| 56 | 75 | 36 ± 5 (54) | 36 ± 4 (43) | 50 ± 4 (24) | 45 ± 4 (33) | 3.3 ± 0.3 (43) | 3.3 ± 0.3 (37) | 4.2 ± 0.2 (30) | 4.3 ± 0.2 (38) |
| 75 | 100 | 19 ± 3 (76) | 31 ± 6 (51) | 51 ± 7 (22) | 37 ± 7 (45) | 2.4 ± 0.2 (58) | 3.2 ± 0.2 (40) | 4.2 ± 0.3 (30) | 3.9 ± 0.3 (43) |
| 150 | 200 | 2 ± 0.3 (98) | 17 ± 3 (73) | | 31 ± 4 (54) | 0.5 ± 0.2 (92) | 2.4 ± 0.2 (53) | | 3.0 ± 0.2 (56) |
| 375 | 500 | | 4 ± 0.5 (93) | | 27 ± 5 (60) | | 1.5 ± 0.1 (71) | | 2.9 ± 0.2 (58) |
| $I_{50}$ value† | | | | | | | | | |
| nmoles/mg protein | | 43 | 54 | ND‡ | 120 | 63 | 140 | ND | 150 |
| $\mu$M | | 57 | 72 | ND | 160 | 84 | 187 | ND | 200 |

* Enzyme activities are reported as the mean ± S. E. M. of duplicative determinations from at least three experiments. Values in parentheses are the per cent inhibition of enzyme activity.

† Calculated from semi-logarithmic plots of the data in this table.

‡ ND indicates that the $I_{50}$ value cannot be accurately determined from the available data in this table.

oxidase activity were studied *in vitro*. Since aminopyrine and aniline are type I and type II substrates, respectively, for the cytochrome P450 mono-oxygenases [15], both compounds were chosen for use in these studies. Aminopyrine *N*-demethylase and aniline hydroxylase activities were inhibited to a varying degree by the four antibacterial agents (Table 2).

Table 3. Summary of spectral changes associated with the interaction of hexachlorophene, tribromsalan, cloflucarban and trichlorcarban with hepatic microsomal cytochrome P 450*

| Compound | $\lambda_{max}$ (nm) | $\lambda_{min}$ (nm) | Binding constant† ($\mu$M) |
|---|---|---|---|
| HCP | 387 | 423 | 33 3 |
| TBS‡ | | 421 | 62 6 |
| TFC | 390 | 424 | 40 4 |
| TCC | 389 | 423 | 66 5 |

* Spectral changes induced by the halogenated antibacterials were determined by difference spectrometry [12].

† The magnitude of absorbance changes at 390 nm minus 420 nm for HCP, TFC and TCC or 422 nm minus 450 for TBS was determined after the addition of ethanolic solutions of different concentrations of the antibacterials to the sample cuvette. Equivalent amounts of ethanol were added to the reference cuvette. The binding constant was determined graphically by plotting the reciprocal of the magnitude of absorbance change versus the reciprocal of the concentration of anitbacterial and by determining the reciprocal of the negative intercept with the abscissa. Each value represents the mean ± S. E. M. of the $K_s$ value determined from three experiments.

‡ An increase in absorbance at about 385–390 nm induced by the binding of TBS to microsomal cytochrome P 450 is obscured by the strong absorbance of the TBS molecule at these wavelengths.

HCP and TBS exhibited more or less conventional linear dose–effect relationships with almost complete inhibition of aminopyrine *N*-demethylase activity occurring at 150 and 375 nmoles/mg of protein respectively. TCC and TFC are less soluble in aqueous media than HCP or TBS, which may explain why no further inhibition of mixed-function oxidase activity could be demonstrated above TCC and TFC concentrations of 30 and 150 nmoles/mg of microsomal protein respectively. Following the comparison of the $I_{50}$ values, aminopyrine *N*-demethylase activity appeared to be more sensitive to the inhibitory effects of these antibacterial agents than did the aniline hydroxylase system.

When the halogenated anitbacterials were added to suspensions of rat hepatic microsomes, spectral changes were observed by difference spectrophotometry. The addition of HCP to rat hepatic microsomes results in a loss of absorbance at about 423 nm concomitant with an increase in absorbance at about 387 nm, which has been termed a type I spectral change [15]. Similar spectral changes were observed for the other halogenated antibacterials, as summarized in Table 2, except that the absorbance of TBS between 340 and 400 nm obscures part of its own binding spectrum. The spectral binding constants of $K_s$ values, each of which signifies the concentration of antibacterial required to obtain half-maximal optical spectral change, are reported in Table 3.

The four compounds had little effect on the flow of reducing equivalents in the microsomal electron transport chain as demonstrated by the lack of inhibition of NADPH–cytochrome *c* reductase activity when microsomes were preincubated with the antibacterials or when these compounds were added directly to the sample cuvette (Table 4). Furthermore, inhibition of microsomal mixed-function oxidase

Table 4. Effect of hexachlorophene, tribromsalan, trichlorcarban and cloflucarban on NADPH–cytochrome *c* reductase activity

| Compound | Concn (nmoles/mg protein) | NADPH–cytochrome *c* reductase activity (nmoles cytochrome *c* reduced/min/mg protein) | Activity (% of control) |
|---|---|---|---|
| Experiment 1* | | | |
| None | 0 | 170 | 100 |
| HCP | 150 | 170 | 100 |
| TCC | 75 | 179 | 105 |
| TBS | 150 | 147 | 87 |
| TFC | 75 | 165 | 97 |
| Experiment 2† | | | |
| None | 0 | 150 | 100 |
| HCP | 750 | 161 | 107 |
| TCC | 750 | 141 | 94 |
| TBS | 750 | 141 | 94 |
| TFC | 750 | 145 | 97 |

* Microsomal protein (4 mg) in 3 ml of tris–HCl buffer, pH 7.4, was preincubated with the halogenated antibacterials for 5 min at 37° and then cooled to 4°. Microsomal protein (0.1 mg) was added to the sample cuvette for the determination of NADPH–cytochrome *c* reductase activity.

† Each antibacterial agent (75 nmoles) was added directly to the sample cuvette, which contained 0.1 mg microsomal protein, immediately prior to the assay of NADPH–cytochrome *c* reductase activity.

activity did not result from impaired NADPH formation since the addition of exogenous NADPH to the incubation reactions, which contained a complete NADPH generating system, did not reverse the inhibitory effect of the antibacterials on mixed-function oxidase activity.

Inhibition of the mixed-function oxidases by the halogenated antibacterials was not linear with respect to time (Fig. 1). Enzyme activity decreased with the time of incubation, suggesting that there was an irreversible inhibition of the aminopyrine *N*-demethylase system.

BSA was added to the incubation medium in an attempt to reverse the inhibition of aminopyrine *N*-demethylase activity produced by the antibacterials, since BSA is known to bind a wide variety of drugs [18, 19], including HCP [6]. One-third of the activity lost in the presence of HCP and TBS was restored by the addition of BSA, while inhibition produced by TFC and TCC was altered only slightly by this protein. BSA did not alter the rate of formaldehyde production in the control incubation reactions.

## DISCUSSION

These studies were designed to investigate the similarities in the effects of chlorinated bisphenols, salicylanilides and carbanilides on the rat hepatic microsomal drug-metabolizing enzyme system. Since each of these compounds appears to exert its antibacterial effect by altering the integrity of biological membranes [20], it was thought that TBS, TFC and TCC might exert effects on mixed-function

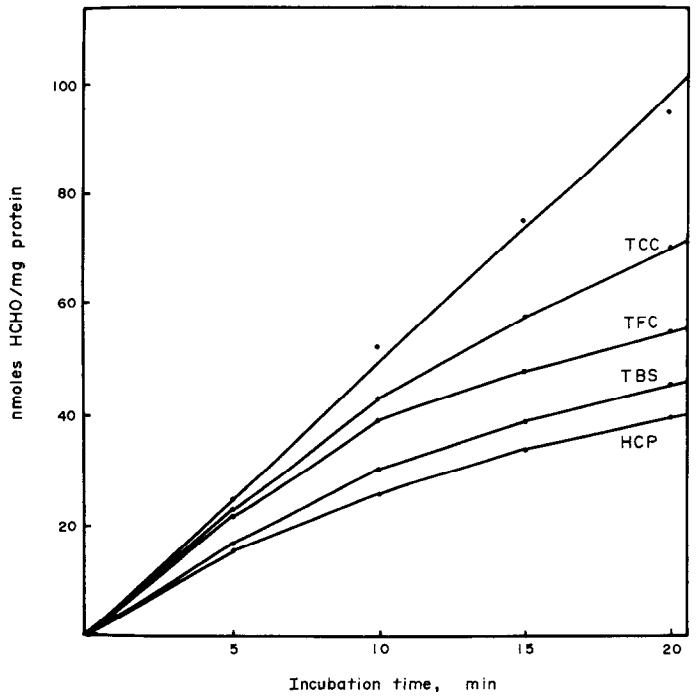


Fig. 1. Time-activity study of the inhibition of aminopyrine *N*-demethylase activity in rat liver microsomes by hexachlorophene, tribromsalan, cloflucarban and trichlorcarban. Rat liver microsomes (4 mg protein) were incubated for varying times with different antibacterial agents: 39 nmoles TCC/mg of protein, 75 nmoles TFC/mg of protein, 75 nmoles HCP/mg of protein and 75 nmoles TBS/mg of protein. Each point represents the average value of two experiments. The top curve represents the control activity.

oxidase activities similar to those previously reported for HCP [6]. This latter antibacterial agent prolonged hexobarbital sleeping time in rats and inhibited mixed function oxidase activity *in vitro*.

It has been reported previously that the maximal effect of HCP on prolonging hexobarbital sleeping time occurs 6 hr after the oral administration of HCP [6]. In the present study, the treatment of rats with TBS and TCC significantly increased hexobarbital sleeping times, while only a slight effect on hexobarbital sleeping times was seen 6 hr after the administration of TFC. Furthermore, when hexobarbital was injected 12 hr after the oral administration of TFC, sleeping times were not prolonged. Although it has been reported that TFC and TCC are absorbed, distributed and eliminated in a similar manner [10], differences in the distribution or metabolism of TFC and TCC *in vivo* could account for the lack of effect of TFC on hexobarbital sleeping time.

These observations suggest that the increased hexobarbital sleeping time after the administration of certain antibacterial agents results from a decreased rate of hexobarbital metabolism, either because of reduced levels of hepatic cytochrome P450 or from a direct inhibition of these enzymes by the antibacterial agents and/or their metabolites. The latter explanation seems more probable since the halogenated antibacterials caused a marked decrease in the hepatic microsomal aminopyrine *N*-demethylase and aniline hydroxylase activities *in vitro*. Furthermore, inhibition of hepatic microsomal drug-metabolizing enzyme activity *in vitro* did not result from an impaired supply of reducing equivalents from NADPH to an acceptor, since NADPH–cytochrome *c* reductase activity was not inhibited by the four antibacterials nor was the inhibition reversed by the addition of exogenous NADPH to the incubation medium.

The spectral binding studies indicate that the inhibition of the hepatic microsomal drug-metabolizing enzyme system may be the result of an interaction of the antibacterials with microsomal cytochrome P450. If it is assumed that the $K_s$ measures the affinity of binding of the substrate or inhibitor molecule to cytochrome P450, then the antibacterial agents have a higher affinity of binding to cytochrome P450 than do aminopyrine and aniline, which have $K_s$ values of 0.33 and 0.36 mM respectively [15]. One possible mechanism of the inhibitory effect of the antibacterial agents on the drug-metabolizing systems is their competition with the drug substrates for binding sites on cytochrome P450.

Inhibitors may block enzyme systems in a number of ways, such as by combining either reversibly or irreversibly with the enzyme, thereby changing the apparent affinity of the enzyme for the substrate or altering the apparent amount of active enzyme in the system. Preliminary kinetic studies indicated that a mixed type of inhibition was seen when the antibacterial agents were added to a microsomal incubation mixture containing aminopyrine as the enzyme substrate. The interpretation of the kinetic studies is difficult since further experiments indicated the presence of irreversible enzyme inhibition.

Time–activity studies were performed to evaluate the effects of the inhibitors. The aminopyrine *N*-demethylase activity in rat liver microsomes decreased with time. At least two possible effects could account for the loss of enzyme activity with time [21] —the inhibitor acts by altering the enzyme irreversibly or the inhibitor is metabolized to a more potent inhibitory metabolite. A reactive metabolite of HCP has been demonstrated to covalently bind to rat liver microsomal protein *in vitro* [22]. One way to check for irreversible enzyme inhibition is to determine whether enzyme activity is regained after dialysis or dilution of the enzyme–inhibitor complex [23]. These methods are not practical when an inhibitor is tightly bound to the enzyme sites, as was thought to be the case in these studies, since the antibacterial agents have a high affinity for microsomal cytochrome P450. Partial protection from the inhibition of aminopyrine *N*-demethylase activity by the antibacterial agents in the presence of BSA could be explained by the binding of the antibacterial agents and their metabolites to BSA rather than to the microsomes.

Although HCP, TBS, TFC and TCC inhibit mixed-function oxidase activity *in vitro* and prolong hexobarbital sleeping time *in vivo* to varying degrees, other factors such as the duration of exposure, the amount of absorption, tissue distribution, and the rate of biotransformation and excretion of each of these agents may be more critical in determining the potential for toxicity after human exposure to these antibacterial agents. The present results suggest that the accidental ingestion or the percutaneous absorption of the various antibacterial agents contained in consumer products may alter the capacity of an individual to metabolize other drugs and chemicals.

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