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PREDICTION OF DRUG–DRUG INTERACTION FROM *IN VITRO* PLASMA PROTEIN BINDING AND METABOLISM

A STUDY OF TOLBUTAMIDE–SULFONAMIDE INTERACTION IN RATS*

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Abstract—The prediction of tolbutamide–sulfonamide interaction from *in vitro* unbound intrinsic clearance was studied by comparing the *in vivo* and *in vitro* total body clearance (CL_{tot}) in rats. The sulfonamides used were sulfaphenazole (SP), sulfadimethoxine (SDM) and sulfamethoxazole (SMZ). The plasma half-life ($T_{1/2}$) of tolbutamide (TB) was increased significantly by SP and SDM, while CL_{tot} was decreased significantly by both drugs; no significant changes were observed in either parameter by SMZ. The *in vitro* plasma protein binding and microsomal oxidation of TB were competitively inhibited by sulfonamides; the order of inhibitor constants was SMZ > SP > SDM. Since metabolism is the rate-determining step of the hepatic extraction of TB, the *in vivo* CL_{tot} can be expressed by the equation: $CL_{tot} \simeq f_B CL_{int}$, where f_B is the blood free fraction and CL_{int} is the hepatic intrinsic clearance of unbound drug. A comparatively good agreement was observed between the *in vivo* CL_{tot} and that calculated from both *in vitro* plasma protein binding and metabolic parameters.

Recent developments in physiologically based pharmacokinetics have made it possible to predict more clearly the clearance of drugs—e.g. good correlations between *in vivo* and *in vitro* metabolic clearances were reported for the deamination of 1-β-D-arabinofuranosylcytosine [1], the oxidation of ethyl alcohol [2], and the demethylation of ethoxybenzamide [3]. Rane *et al.* [4] also reported that good agreement was obtained between the hepatic extraction ratio predicted from *in vitro* intrinsic clearance and that observed in the isolated perfused rat liver, for antipyrine, carbamazepine, hexobarbital, phenytoin, propranolol, alprenolol and lidocaine, which span extraction ratios from less than 0.1 to more than 0.9. However, little has been reported of the physiologically based pharmacokinetics of drug–drug interaction of plasma protein binding and metabolism.

The drug interaction of tolbutamide (TB), a sulfonylurea derivative, with a second drug—e.g. sulfonamide (SA)—has been extensively studied, suggesting two possible mechanisms, i.e. displacement of plasma protein binding and metabolic inhibition [5–10]. Previous *in vivo* experiments in this laboratory have demonstrated that the strong binding tendency of sulfaphenazole to microsomal cytochrome P-450 at the type II binding site might explain the metabolic inhibition of TB that was observed in the elimination of TB in the presence of sulfaphenazole in rats [11].

The purpose of this study was to predict the effect of SA on the elimination of TB in rats by comparing the total body clearance (CL_{tot}) obtained from both *in vivo* and *in vitro* experiments in an attempt to elucidate the mechanism of the drug interaction of TB with SA. Sulfonamides employed were sulfaphenazole (SP), sulfadimethoxine (SDM) and sulfamethoxazole (SMZ).

MATERIALS AND METHODS

Sodium tolbutamide (TB), sodium sulfadimethoxine (SDM) and sodium sulfamethoxazole (SMZ) were supplied by Japan Hext (Tokyo, Japan), the Daiichi Pharmaceutical Co. (Tokyo, Japan), and the Shionogi Pharmaceutical Co. (Ohsaka, Japan) respectively. Sulfaphenazole (SP) was obtained from the Dainippon Pharmaceutical Co. (Tokyo, Japan). NADPH was purchased from Boehringer Mannheim GmbH, Mannheim, West Germany. [^{14}C-Carbonyl]TB (48.09 mCi/mmole) was purchased from the New England Nuclear Corp. (Boston, MA) and was found to be at least 98–99 percent pure by t.l.c. Hydroxytolbutamide (HTB) was synthesized by the method of Thomas and Ikeda [12]. All other reagents were commercially available and of analytical grade.

Animal experiments. Adult male Wistar rats (Nihon Seibutsu Zairyo, Tokyo, Japan) weighing 260–280 g were used. Under light ether anesthesia, the femoral vein and artery were cannulated with PE-10 and PE-50 polyethylene tubing respectively. Cannulated rats were kept in restraining cages with water under normal housing conditions prior to the experiments. After loading doses of 160, 200 and

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200 mg/kg, SP, SDM and SMZ, respectively, at doses of 50.0, 41.3 and 41.3 mg/kg per hr, respectively, were infused through the femoral vein cannula for 6 hr with a constant rate infusion pump (Natsume Seisakusho Co., model KN, type 12H, Tokyo, Japan); with these dosages, steady-state concentrations of SA (500 μg/ml) were obtained within 20–35 min after the beginning of the infusion. At 50 min after the initiation of infusion, the rats were given 80 mg/kg of TB containing 3.33 μCi/kg of [^{14}C]TB in physiological saline through the other femoral vein cannula; blood samples (0.25 ml) were then obtained at 0.25, 0.5, 1, 2, 3, 4, 5 and 6 hr, in heparinized polyethylene centrifuge tubes (Beckman Instruments, Fullerton, CA). The body temperature was kept at 37° by a heat lamp. Plasma was separated by centrifugation for 20 sec in a table-top microfuge (Beckman Instruments). The separation of the metabolites from TB was carried out according to Shibasaki *et al.* [13]. Briefly, 50 μl of the plasma sample was added with 3 ml of 0.5 M phosphate buffer (pH 5.0), and the mixture was shaken for 30 min and then extracted twice with 6 ml of *n*-heptane–chloroform (4:1, v/v). The extracts were combined and shaken with 1 ml of 0.5 N NaOH for 15 min and the aqueous phase was used for the determination of TB. The extraction efficiency of TB from plasma was 0.77 ± 0.05 (N = 3). The negative interference by HTB was verified by pure HTB. The concentration of ^{14}C-labeled TB was determined in an Aloka Tri-Carb counter (Aloka Instruments Co., Tokyo, Japan) after 0.5 ml of the aqueous phase was placed in a scintillation vial containing 0.5 ml of 0.5 N HCl and 10 ml of scintillation fluid (0.1 g of POPOP, 4.0 g of PPO and 500 ml of Triton X-100/liter of toluene).* The concentration of each SA in plasma was determined by the method of Tsuda and Matsunaga [14]. No interference with TB and its metabolites was confirmed in the determination of SA.

Plasma protein binding. Plasma protein binding was determined by an ultrafiltration method using a membrane cone (Amicon Centriflo Ultrafiltration Membrane Filter Cone, type CF-25, Lexington, MA), which provides 10 percent of the initial plasma volume after centrifugation at 1000 *g* for 3 min. One milliliter of plasma sample containing 0.1 to 0.2 mM [^{14}C]TB (200 nCi) and 500 μg/ml of SA was applied to the membrane cone after incubation at 37° for 5 min. The concentrations of ^{14}C-labeled TB and each SA were determined as described above. The adsorption of ^{14}C-labeled TB to the membrane was negligible.

Oxidation in microsomes. Rats were killed by cutting the carotid artery. The liver was perfused through the inferior vena cava with ice-cold 0.9% NaCl for 2 min. Immediately thereafter, the liver was removed and homogenized in 3 vol. of ice-cold 0.9% NaCl in a teflon–glass homogenizer. The homogenate was centrifuged at 4° for 15 min in a refrigerated centrifuge (Hitachi 20PR-5, Hitachi Koki Co. Ltd., Tokyo, Japan) at 9,000 *g*. The supernatant fraction was then centrifuged at 77,000 *g* for 90 min in a Hitachi 65P ultracentrifuge (Hitachi Koki Co. Ltd.) at 4°. The microsomal pellets were suspended in a volume of ice-cold 0.9% NaCl to make a concentration equivalent to 30 mg of wet tissue weight/ml. The final reaction mixture contained 100 nCi/ml of [^{14}C]TB and various amounts of nonlabeled TB, 6 mg of microsomal suspension, 5 μmoles of $MgCl_2$, 0.1 μmole of EDTA, and 1 μmole of NADPH in 1 ml of 0.05 M Tris–HCl buffer (pH 7.4). The TB concentration ranges were 0.02 to 0.66 mM. The SP, SDM and SMZ concentrations in the reaction mixture were 0.77, 0.69 and 1.67 mM, respectively, and these concentrations were in the same range as those of the *in vivo* steady-state free concentration of each SA in plasma. The reaction was started by adding NADPH to the incubation medium after 2 min of preincubation at 37°. The mixture was shaken at 37° for 3 min, and the reaction was stopped by addition of 1 ml of 1 N HCl. The reaction was linear for 4 min, and it was directly related to the concentration of microsomal protein in the reaction mixture at a concentration of 6–8 mg/ml. The resultant HTB was determined from measurement of radioactivity after the extraction. The incubation mixture was shaken with 8 ml of ethyl ether for 20 min. After centrifugation, 7 ml of the ether layer was shaken with 0.5 ml of 0.5 N NaOH for 20 min and then centrifuged for 10 min; the extraction of HTB was carried out by the method of Martin and Rowland [15] after slight modifications. The aqueous phase was adjusted to pH 5.0 by 2.55 M NaH_2PO_4 and was extracted three times with 8 ml of *n*-heptane [containing 0.5% (v/v) isoamyl alcohol] to remove more than 99 percent of TB. The concentration of [^{14}C]HTB was determined after an aliquot (0.1 ml) of the aqueous layer was placed in a scintillation vial containing 0.9 ml of distilled water and 10 ml of scintillation fluid as described above. The procedural recovery of HTB determined by an ultraviolet method was 0.95 ± 0.01 (N = 15). The correction for the procedural loss was made in all experiments. The amount of cytochrome P-450 was determined by the method of of Omura and Sato [16, 17]. Nonspecific binding to microsomes was determined by using the above enzyme reaction mixture, omitting NADPH. The mixture was ultrafiltered, using a membrane cone in the same manner as described in the section on "Plasma Protein Binding". The protein concentration was determined by the biuret method using bovine serum albumin as the standard [18]. The inhibitor constant for the metabolism was calculated by equations 2–6 of Appendix 1.

Plasma-to-blood concentration ratio of TB. All the precedures were carried out right after blood collection. The blood was incubated with 100 nCi/ml of [^{14}C]TB and various amounts of unlabeled TB (0.1 to 1.5 mM) at 37° for 20 min in the presence of SA. The concentrations of SP, SDM and SMZ in blood were 1.60, 1.42 and 1.80 mM (corresponding to 1.60, 1.60 and 1.97 mM in plasma) respectively. After centrifugation, an aliquot of the plasma was removed and the concentration of [^{14}C]TB was determined as described above. An analytical blank without substrate was determined in the same manner. Hemolysis during the incubation was negligible.

* POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazo-lyl)]-benzene; and PPO = 2,5 diphenyloxazole.

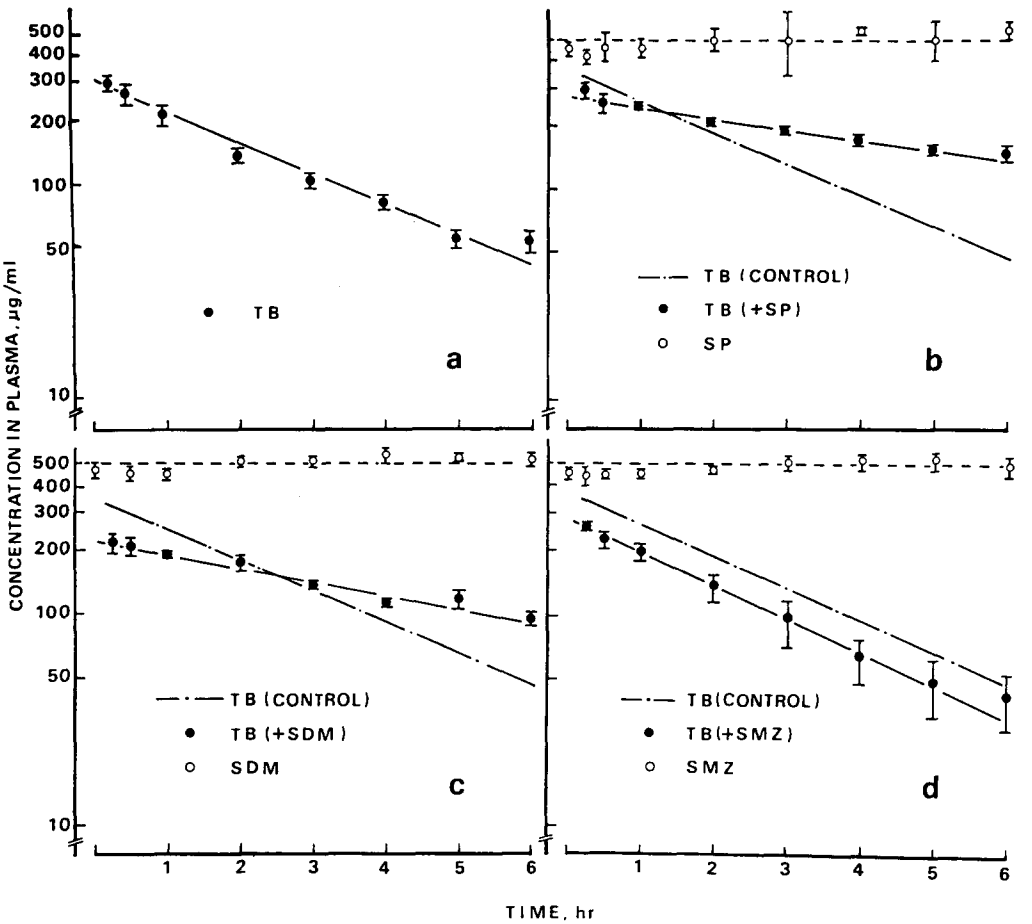

Fig. 1. Plasma disappearance of tolbutamide (TB) after intravenous administration of 80 mg/kg in rats with and without sulfonamides (SA). Each point and vertical bar represents the mean and S.E. of three rats. Lines were calculated by the "SALS" method [19] using a digital computer. Panel a: control (without SA); panel b: with SP (C_{ss} = 493.4 ± 33.3 μg/ml; N = 3); panel c: with SDM (516.8 ± 5.8; N = 3); and panel d: with SMZ (494.3 ± 5.7; N = 3); Key: (—●—) TB; (--○--) SA; and (———, panels b, c and d), TB concentration of the control rats as shown in panel a.

Data analysis. The data were analyzed by an iterative least squares method using a Hitachi 8700/8800 digital computer. The program used was SALS [19] for the mono-exponential curve fitting, the Michaelis–Menten equation, and the calculation of the binding parameters of Scatchard plots respectively.

Statistical analysis. All means are presented with their standard errors (the mean ±S.E.). Student's *t*-test was utilized to estimate a significant difference between the control and the sulfonamide groups.

Table 1. *In vivo* pharmacokinetic parameters of tolbutamide in rats*

	Constant					
	C_O (μg/ml)	β (min^{-1})	$T_{1/2}$ (hr)	Vd_β (ml/kg)	AUC† [(mg/ml) × min]	Total body clearance‡ ($ml \cdot min^{-1} \cdot kg^{-1}$)
Control	369.16 ± 3.59	0.0063 ± 0.0002	1.83 ± 0.06	216.76 ± 2.13	58.29 ± 1.43	1.37 ± 0.03
+SP	269.00 ± 5.75	0.0020 ± 0.0002§	5.97 ± 1.73§	297.79 ± 6.46§	138.72 ± 9.66§	0.59 ± 0.04§
+SDM	212.69 ± 5.39	0.0023 ± 0.0003§	5.37 ± 0.73§	376.83 ± 9.88§	97.81 ± 10.47‖	0.84 ± 0.08§
+SMZ	279.39 ± 6.14	0.0062 ± 0.0077	1.94 ± 0.13	286.73 ± 6.32§	46.74 ± 4.79	1.77 ± 0.19

* Results are the means ± S.E. of three rats. Dose: 80 mg/kg, i.v.
† Calculated by the equation: $AUC = C_O/\beta$.
‡ Calculated by the equation: total body clearance (CL_{tot}) = dose/AUC.
§ Significantly different ($P < 0.01$) from the control.
‖ Significantly different ($P < 0.05$) from the control.

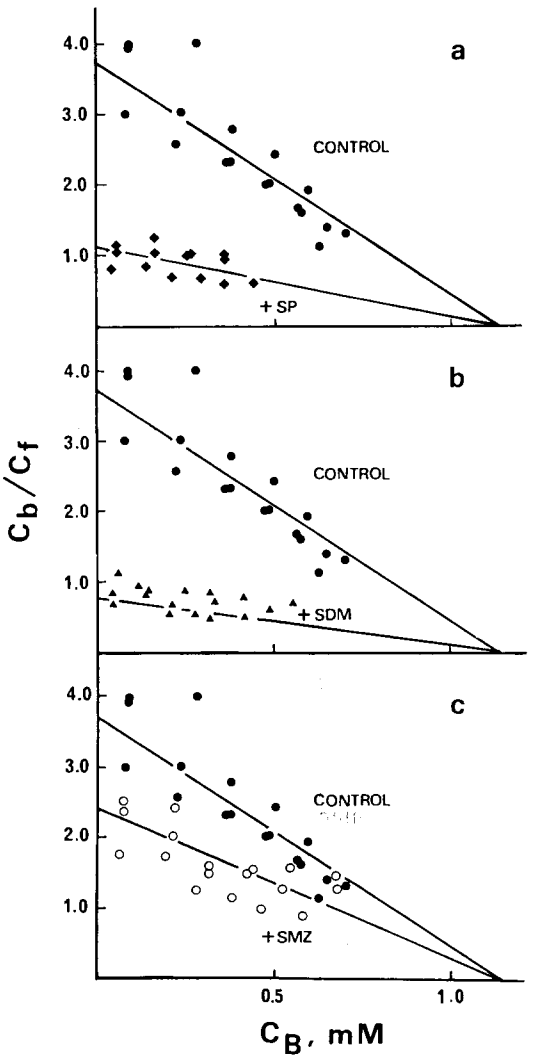

Fig. 2. Scatchard plots of data for the binding of TB to rat plasma. Lines were fitted by a least squares regression by the "SALS" method using a digital computer [19]. See Materials and Methods for details. Key: (●) control (without SA); (◆) with SP (panel a); (▲) with SDM (panel b); and (○) with SMZ (panel c).

RESULTS

Effect of sulfonamides on TB elimination from plasma. The plasma disappearance of TB, after intravenous administration of 80 mg/kg, with and without the presence of SA is shown in Fig. 1, panels a, b, c and d. The disappearance of TB followed mono-exponential curves in both the control and SA-treated rats. The plasma concentrations of SA were kept at a constant level of about 500 μg/ml during the sampling period for 6 hr. The plasma half-lives ($T_{1/2}$) of TB increased significantly from 1.83 ± 0.06 hr for the control rats (Fig. 1a) to 5.97 ± 1.73 hr for the SP-treated rats ($P < 0.01$) (Fig. 1b), and to 5.37 ± 0.73 hr for the SDM-treated rats ($P < 0.01$) (Fig. 1c), but no significant difference was observed between the control and SMZ-treated rats, which had a $T_{1/2}$ of 1.94 ± 0.13 hr (Fig. 1d). The pharmacokinetic constants were computed by a non-linear iterative least squares method [19] and are listed in Table 1. In both the SP- and SDM-treated rats, a significant decrease was observed in the plasma total body clearance (CL^{p}_{tot}), while in the SMZ-treated rats, CL^{p}_{tot} did not show a significant alteration. The distribution volume, Vd_{β} showed a significant increase by all sulfonamides studied.

Table 2. Binding parameters of tolbutamide to plasma, determined by an ultrafiltration method*†

	$n(p)$ (mM)	K_d (mM)	I_f (mM)	K_I (mM)
Control	1.13	0.300		
+SP	1.13	1.010‡	0.77	0.325
+SDM	1.13	1.149‡	0.69	0.243
+SMZ	1.13	0.471‡	1.67	2.929

* Values were calculated from the results shown in Fig. 3.
† See Materials and Methods and Appendix.
‡ Apparent K_d was calculated from I_f and K_I.

Effect of sulfonamides on plasma protein binding of TB. Scatchard plots of TB binding to plasma protein obtained from ultrafiltration are shown in Fig. 2, panels a, b and c. In both the experiments, i.e. with and without sulfonamides, there was no evidence for the existence of more than one class of binding site in the concentration range tested, which corresponds to the *in vivo* plasma concentration range of TB after intravenous administration of 80 mg/kg. The binding parameters with and without the presence of sulfonamides were calculated by a non-linear iterative least squares method [19] without the constraint of parameters. A typical competitive inhibition of TB binding was observed for all sulfonamides tested (Fig. 2). The average value of $n(p)$ was 1.13 ± 0.05 mM (mean ± S.E. of four experiments). The apparent dissociation constant (K_d) in the presence and absence of sulfonamide was recalculated for each experiment corresponding to the constrained value of $n(p)$ (1.13 mM). As shown in Table 2, the K_d of TB increased from 0.3 mM for the control to 1.01, 1.15 and 0.47 mM in the presence of SP, SDM, and SMZ respectively. The free concentrations of TB (I_f) at 500 μg/ml of the total concentration of sulfonamides were 0.77, 0.69, and 1.67 mM for SP, SDM, and SMZ respectively. The

Table 3. Plasma-to-blood concentration ratio of tolbutamide*†

	C_p/C_B
Control	1.33 ± 0.08
+SP	1.27 ± 0.06
+SDM	1.23 ± 0.07
+SMZ	1.37 ± 0.01

* Results are the mean ± S.E. of three experiments.
† See Materials and Methods.

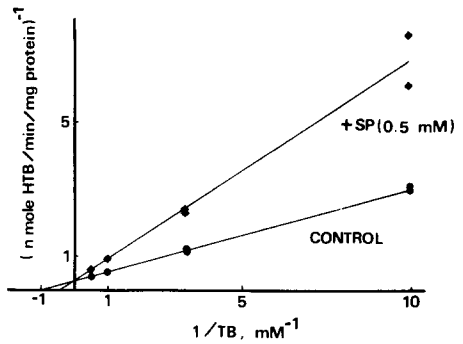

Fig. 3. Lineweaver–Burk plots of the appearance rate of HTB versus the initial TB concentration. The plots were analyzed by an iterative least squares method using a digital computer [19]. Key: (●) control (without SA); and (◆) with SP (0.5 mM).

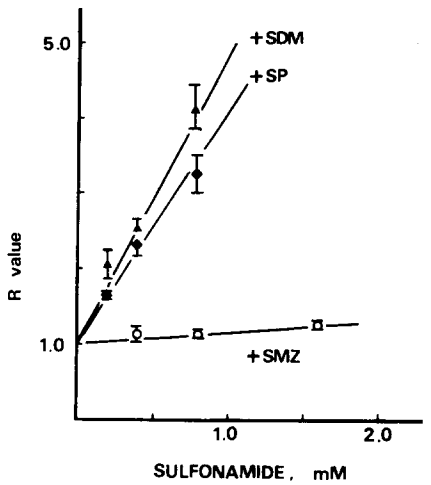

Fig. 4. Relationship between the ratio (R) of the *in vitro* metabolic rate of TB with (v') and without (v) SA and the free concentration (I_f) of SA. See Appendix 1 for details.

order of inhibitor constants of sulfonamides for the plasma protein binding of TB was SMZ > SP > SDM.

Plasma-to-blood concentration ratio of TB. The ratios of plasma-to-blood concentrations of TB in the dose range from 0.1 to 1.5 mM were determined in the presence and absence of sulfonamides and are listed in Table 3. The ratios seem to be constant in the dose ranges studied.

Effects of sulfonamides on hydroxylation of TB by liver microsomes. Lineweaver–Burk plots of the appearance rate of HTB versus the initial substance concentration are shown in Fig. 3. The plots were analyzed by an iterative least squares method using a digital computer [19]. In the presence of SP, a typical competitive inhibition of the hydroxylation of TB was observed. The inhibitions by both SDM and SMZ, although not shown in the figures, were also competitive. The calculated *in vitro* metabolic parameters are listed in Table 4. The V_{max} was 2.16 nmoles HTB formed·min^{-1}·(mg microsomal protein)$^{-1}$, and in order to use this value for the calculation of the V_{max} of the whole liver (V_{max}^{WL}), the following correction was carried out. The cytochrome P-450 content in the liver microsomes determined in this study (0.62 ± 0.03 nmole/mg microsomal protein) was comparable with those of the previous reports [20–24]. The amount of cytochrome P-450 content per gram of rat liver has been reported to be 30.0 [20], 29.1 [21], 30.0 [22], 34.9, 36.9, 28.3 [23], and 47.5 [24]. Since these values have small variations, the mean of these values (33.8 ± 2.6; N = 7) was used for the calculation. The V_{max}^{WL} was calculated by use of the following equation 1.

$$V_{max}^{WL} = \frac{V_{max}^{in\,vitro}\ (\text{nmoles/mg protein/min}) \times 33.8 \times 12.4\ (\text{wet liver weight of 280 g rat})}{0.62} \quad (1)$$

Table 4. *In vitro* metabolic parameters of tolbutamide

	V_{max}*	V_{max}†	V_{max}‡	$K_{m_{app}}$§	f_{Ms}‖	K_m¶	I_{tot}**	f_{SA}††	I_f‡‡	K_i§§	K_m‖‖
Control	2.16	0.12	1.44	1.20	0.80	0.96					
+SP	2.16	0.12	1.44		0.85		1.60	0.48	0.77	0.30	3.42
+SDM	2.16	0.12	1.44		0.85		1.60	0.43	0.69	0.24	3.75
+SMZ	2.16	0.12	1.44		0.87		1.97	0.85	1.67	6.69	1.20

* Expressed as nmoles HTB formed·min^{-1}·(mg microsomal protein)$^{-1}$.

† Expressed as μmoles HTB formed·min^{-1}·(g liver wet weight)$^{-1}$ (see Results).

‡ Expressed as μmoles HTB formed·min^{-1}·(280 g rat)$^{-1}$. The value of 12.4 g was used as the liver wet weight per rat.

§ The apparent K_m value (mM).

‖ The free fraction of tolbutamide in the incubation mixture (see Materials and Methods).

¶ The K_m value calculated from the $K_{m_{app}}$ by multiplying the f_{Ms} (mM).

** The total concentration of sulfonamide in plasma (mM).

†† The free fraction of sulfonamide in plasma.

‡‡ The plasma free concentration of sulfonamide calculated from the I_{tot} and f_{SA} (mM).

§§ The inhibition constant (mM) (see Appendix).

‖‖ Calculated from the following equation:

$$K_m = K_m' \left(1 + \frac{I_f}{K_i}\right)$$

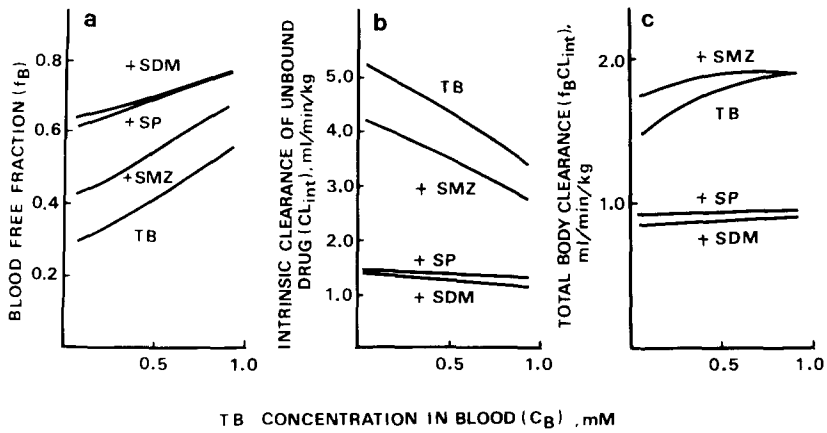

Fig. 5. Simulation curves for blood free fraction (f_B), intrinsic clearance of unbound drug (CL_{int}) and total body clearance ($CL_{tot} \simeq f_B CL_{int}$) as a function of the blood total concentration (C_B) of TB with and without sulfonamides. Panel a: f_B; panel b: CL_{int} and panel c: CL_{tot} ($f_B CL_{int}$).

In order to calculate the inhibition constant (K_i) of sulfonamides for the TB oxidation, the plots of the ratio of v to v' (R) versus I_f in equation 4 of Appendix 1, were carried out, and straight lines with a positive intercept ($R = 1.0$) were obtained (Fig. 4). The order of inhibitor constants of SA for the oxidation metabolism of TB was SMZ > SP > SDM. The apparent K_m values in the presence of SA were calculated by I_f(‡‡), K_i(§§) and K'_m(¶). and are listed in Table 4.

Blood concentration dependency of blood free fraction of TB, and intrinsic clearance of unbound and total TB. Using the values of 0.025 to 0.4 mM for C_f of TB, which was the range of the free concentration in the blood after intravenous administration, the CL_{int} and f_B were calculated by equations 9 and 11 of Appendix 2 respectively. The C_B was also calculated from the ratio of C_f to f_B. The simulation curves for C_B vs f_B, C_B vs CL_{int} and C_B vs $f_B \cdot CL_{int}$ ($\simeq CL_{tot}$) are shown in Fig. 5, panels a, b and c respectively. In the control without SA, simulation curves showed the blood concentration dependency of plasma binding (f_B), metabolism (CL_{int}) and intrinsic clearance ($f_B \cdot CL_{int}$) (Fig. 5, panels a and b). In the presence of SDM and SP, these concentration dependencies of TB disappeared, whereas SMZ did not show a remarkable effect. This might be explained by the weak inhibitory effects of SMZ on both the plasma binding and metabolism of TB.

Relationship between in vivo *and* in vitro *total body clearance.* The *in vivo* and *in vitro* CL_{tot} were calculated by equations 7–13 in Appendix 2. To calculate the *in vitro* CL_{tot}, the C_f of the *in vivo* mean plasma concentration ($\overline{C}_t$) for 6 hr (360 min) was used. The $\overline{C}_t$ was calculated mathematically by an equation: $\overline{C}_t = AUC_{0 \to 360\,min}/360$ min, where AUC is the area under the plasma concentration time curve, using the pharmacokinetic parameters listed in Table 1. The relationship between the *in vivo* and *in vitro* CL_{tot} is shown in Fig. 6.

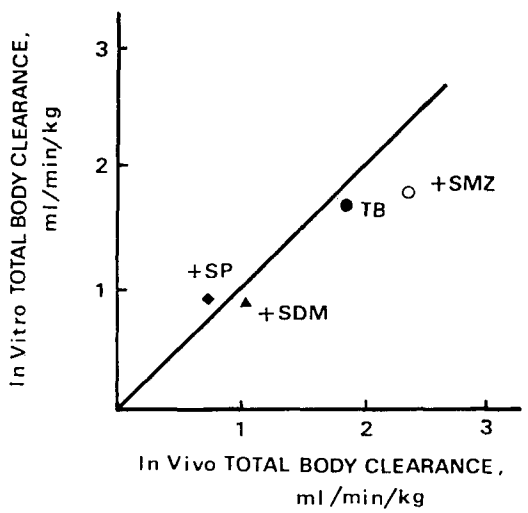

Fig. 6. Relationship between *in vivo* and *in vitro* total body clearance (CL_{tot}). The line shows a positive correlation ($r = 1.000$).

DISCUSSION

When metabolism is the rate-determining step of hepatic extraction, the total body clearance (CL_{tot}) can be expressed by equation 8 in Appendix 2.

$$CL_{tot} \simeq f_B \cdot CL_{int} \quad (8)$$

The relationship of equation 8 shows that both the increase of f_B due to the displacement by the second drug and the change of CL_{int} due to metabolic inhibition or induction may affect CL_{tot}. The results of this investigation demonstrate that the acute interaction between TB and SA in rats involved two possible mechanisms, namely the displacement of TB from the binding sites of the plasma protein by SA (Table 2, Fig. 2) and the inhibition of the CL_{int} of TB (Fig. 3, Table 4). These effects were produced at the average concentration of SA in plasma (500 μg/ml) which was in the same range as previously reported for thiopental–SDM interaction in rats [25].

TB is eliminated mainly by metabolism to hydroxytolbutamide (HTB) [11, 26]. The concentration of HTB observed in both the control and SP-treated rats was less than one-tenth of that of TB [11]. The concentrations of the major metabolite, N^4-acetyl SA in rat plasma, were 9.6 ± 0.6% of the total amount (SA + N^4-acetyl SA) for SP, 7.3 ± 0.6% for SDM, and 15.6 ± 2.0% for SMZ respectively. These findings suggest that both the metabolites of TB and SA may not affect primarily the protein binding and the metabolism of TB.

An increase in the plasma unbound fraction of TB was observed after the addition of SA (500 μg/ml) (Fig. 5a), and this observation was in agreement with the results of Thiessen and Rowland [10] that SDM displaced TB from the binding sites of sheep plasma, and also with those of Judis [27] that demonstrated the displacement of TB from the binding sites of human serum albumin by SP and SDM. In the presence of SA, a typical competitive inhibition of the hydroxylation of TB was also demonstrated (Table 4), although previously the competitive binding of TB and SA to rat microsomes was suggested by difference spectrum [11].

The intra- and extracellular pH difference in the hepatocyte has been reported to be about 0.3 [28], and the pK_a values of TB, SP, SDM and SMZ are 5.43 [13], 6.09, 6.32 and 6.05 [29] respectively. Consequently, if the sinusoidal plasma membrane of the hepatocyte is impermeable to the ionized drugs and follows perfectly the pH partition theory, the intracellular free concentrations of both TB and SA should be corrected. However, in this study a relatively good correlation was revealed between *in vivo* and *in vitro* CL_{tot} (Fig. 6), suggesting that the sinusoidal plasma membrane of the hepatocyte might not follow the pH partition theory. The validity of this hypothesis in the uptake of TB by the hepatocytes is now under investigation using isolated rat hepatocytes.

From the results of Fig. 6, it can be seen that *in vitro* CL_{tot} in the control rat was very close to *in vivo* CL_{tot}, while *in vitro* CL_{tot} values in the presence of SP, SDM and SMZ were within 0.76 to 1.26 times those of *in vivo* CL_{tot}. This variation might be due to the effectiveness of pH-dependent distribution of TB *in vivo* and/or the inhibition of plasma protein binding and metabolism by N^4-acetyl SA. However, since the difference of CL_{tot} between *in vivo* and *in vitro* was not large, the present results suggest that the enzyme kinetic parameters obtained from *in vitro* studies might be useful for the calculation of *in vivo* CL_{tot}. From these findings, it was suggested that the parameters for plasma protein binding and the enzyme kinetic parameters for microsomal oxidation from *in vitro* studies could be adapted to predict the approximation of the *in vivo* total body clearance in the presence of a drug–drug interaction.

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GLOSSARY

CL_{tot}	blood total body clearance, ml · min^{-1} · kg^{-1}
CL_{int}	intrinsic clearance of unbound drug, ml · min^{-1} · kg^{-1}
Q_h	hepatic blood flow, ml/min
C_f	free concentration of TB, mM
C_b	bound concentration of TB, mM
C_p	plasma total concentration of TB, mM
C_B	blood total concentration of TB, mM
AUC_p	area under plasma concentration time curve, (mg/ml) · min^{-1}
s	ratio of C_p to C_B
f_p	plasma free fraction of TB
f_B	blood free fraction of TB

n	number of binding sites
(p)	concentration of plasma protein, mM
K_d	dissociation constant of plasma protein binding, mM
K_I	inhibitor constant of sulfonamide for TB plasma protein binding, mM
I_f	free concentration of sulfonamide, mM
v	oxidative metabolic rate of TB, μmoles HTB·min^{-1}·(12.4 g liver)$^{-1}$
v'	oxidative metabolic rate of TB in the presence of sulfonamide, μmoles HTB·min^{-1}·(12.4 g liver)$^{-1}$
V_{max}	maximum velocity, μmoles HTB·min^{-1}·(12.4 g liver)$^{-1}$
K_m	Michaelis constant, mM
K_i	inhibitor constant of sulfonamide for oxidative metabolism of TB, mM
R	ratio of v to v'
α	slope of the regression line obtained from the plots of R versus I_f

APPENDIX 1: CALCULATION OF INHIBITOR CONSTANT

The inhibitor constant (K_i) of sulfonamide for the TB oxidation was calculated as follows:

The metabolic rate of TB is given by equation 2:

$$v = \frac{V_{max} \cdot C_f}{K_m + C_f} \tag{2}$$

In the presence of sulfonamide which inhibits competitively the TB oxidation, equation 2 is modified to equation 3:

$$v' = \frac{V_{max} \cdot C_f}{K_m \cdot \left(1 + \frac{I_f}{K_i}\right) + C_f} \tag{3}$$

From equations 2 and 3, equation 4 was obtained for R:

$$R = \frac{v}{v'} = 1 + \frac{\frac{K_m}{K_i}}{K_m + C_f} I_f \tag{4}$$

A plot of R with its corresponding I_f yields a straight line with a positive intercept, and the slope (α) is given by equation 5:

$$\alpha = \frac{K_m}{K_i \cdot (K_m + C_f)} \tag{5}$$

The slope was calculated by an iterative least squares method "SALS" [19] using a Hitachi 8700/8800 digital computer. The intercept was fixed at 1. Thus, the inhibition constant (K_i) can be calculated by equation 6:

$$K_i = \frac{K_m}{\alpha \cdot (K_m + C_f)} \tag{6}$$

In this study, we used the values of 0.08 mM for C_f and 0.96 mM for K_m respectively.

APPENDIX 2: CALCULATION OF TOTAL BODY CLEARANCE

In vivo total body clearance (CL_{tot}) is given by equation 7:

$$CL_{tot} = \frac{Q_h \cdot f_B \cdot CL_{int}}{Q_h + f_B \cdot CL_{int}} = \frac{\text{Dose}}{AUC_p}\left(\frac{C_p}{C_B}\right) = \frac{\text{Dose}}{AUC_p} \cdot s \tag{7}$$

Since the CL_{tot} of TB is 0.4 ml·min^{-1}·(280 g body weight)$^{-1}$ and is considerably smaller than the hepatic blood flow (Q_h), 16.5 ml·min^{-1}·rat^{-1} [30], we can assume the intrinsic clearance limited as expressed by equation 8:

$$CL_{tot} \simeq f_B \cdot CL_{int} \tag{8}$$

and in the plasma protein binding, the plasma free fraction also depends on the concentration of TB, and if we assume a competitive inhibition by sulfonamide for the plasma protein binding of TB, the plasma free fraction of TB (f_p) can be expressed by equation 9:

$$f_p = \frac{C_f}{C_p} = \frac{C_f}{C_f + \frac{n \cdot (p) \cdot C_f}{K_d \cdot \left(1 + \frac{I_f}{K_I}\right) + C_f}} \tag{9}$$

The relation of the blood free fraction (f_B) and f_p is given by equation 10:

$$f_B = \frac{C_f}{C_B} = f_p \cdot \left(\frac{C_p}{C_B}\right) = f_p \cdot s \tag{10}$$

From equations 9 and 10 we obtain equation 11:

$$f_B = \frac{s}{1 + \frac{n \cdot (p)}{K_d \cdot \left(1 + \frac{I_f}{K_I}\right) + C_f}} \tag{11}$$

On the other hand, the CL_{int} of TB depends on the concentration of TB, and if a competitive inhibition by SA for the CL_{int} of TB is assumed, the CL_{int} can be expressed by equation 12:

$$CL_{int} = \frac{V_{max}}{K_m\left(1 + \frac{I_f}{K_i}\right) + C_f} \tag{12}$$

Thus, from equations 8, 11 and 12, the following equation 13 is obtained for the CL_{tot} of TB:

$$CL_{tot} = \frac{s \cdot V_{max}}{\left[1 + \frac{n \cdot (p)}{K_d\left(1 + \frac{I_f}{K_I}\right) + C_f}\right]\left[K_m\left(1 + \frac{I_f}{K_i}\right) + C_f\right]} \tag{13}$$

In this study, using the values of 0.025 to 0.40 mM for C_f, which is the same range of C_f in blood after intravenous administration of TB, the CL_{int} and the f_B were calculated by equations 9 and 11 respectively. The C_B was calculated using the ratio of C_f to f_B. Then, the relations of C_B vs f_B (Fig. 5a), C_B vs CL_{int} (Fig. 5b), and C_B vs CL_{tot} (Fig. 5c) were simulated.