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DECREASED IN VIVO ACETYLATION OF SULFISOXAZOLE IN THE RABBIT IN THE PRESENCE OF 4-N-ACETYL-SULFISOXAZOLE

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SUMMARY

When 4-N-acetyl-sulfisoxazole, the primary metabolite of sulfisoxazole, is allowed to accumulate by continuous infusion, a decrease in the plasma protein binding, tissue protein binding and metabolism of sulfisoxazole is observed. A 5-fold decrease in the unbound metabolic clearance, which represents the intrinsic ability of the rabbit to acetylate sulfisoxazole, supports the idea that product inhibition takes place. However, this decrease in the metabolism appears to be much less pronounced when the total metabolic clearance is considered because the effect is partially obscured by decreased plasma protein binding.

INTRODUCTION

Several metabolites formed by oxidation via the P450 system are known to inhibit the oxidation of other compounds (Jähnchen and Levy, 1972; Ashley and Levy, 1973; Klotz et al., 1976). This inhibition presumably occurs by competitive binding for the same enzyme site. Such competition is less well documented for acetylation. In vitro studies of isoniazid have demonstrated that an acetylated metabolite may inhibit its own formation by interaction with N-acetyltransferase (Weber, 1973). Studies of procainamide in renal failure patients where N-acetyl-procainamide accumulates (Gibson et al., 1977) may be interpreted to support the notion that acetylated compounds can act as product inhibitors in vivo. In our recent report on sulfisoxazole elimination in the rat in the presence of high plasma concentrations of 4-N-acetyl-sulfisoxazole, we concluded that the metabolic formation, as well as renal elimination, were decreased in comparison to the absence of metabolites (Øie, 1975). However, these conclusions assume that: (1) the difference

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between the total clearance and the observed renal clearance multiplied by the plasma sulfisoxazole concentration is equal to the formation rate of 4-N-acetyl-sulfisoxazole; (2) that the protein binding of sulfisoxazole found after termination of the kinetic studies was representative of the binding during the studies; and (3) that the urine pH was constant in the cross-over studies thereby maintaining a constant reabsorption of sulfisoxazole in the kidney.

In order to determine if the renal and/or metabolic elimination of sulfisoxazole is affected by 4-N-acetyl-sulfisoxazole without having to make the assumptions above, we have carried out kinetic studies using radioactive sulfisoxazole in the presence and absence of 4-N-acetyl-sulfisoxazole administration in the rabbit. This permitted direct measurement of the amount of metabolite formed. The rabbit also allowed for continuous measurement of unbound concentration of sulfisoxazole in plasma throughout the studies as sufficient blood could be obtained without rendering the animal anemic. The urine pH was maintained constant with an infusion of a sodium bicarbonate solution.

The acetylation was appreciably decreased in the presence of 4-N-acetylsulfisoxazole. In contrast to our rat studies, however, we found the renal elimination to be unaffected.

METHODS AND MATERIALS

Animals Twelve New Zealand white male rabbits weighing 2.1–4.0 kg were studied. Before each experiment the marginal ear-vein of each ear was cannulated with an Intracath catheter (22-gauge). The urethra was cannulated with a French Foley no. 8 catheter. The animals were placed in a plastic rabbit-restraining cage for the duration of the experiment. No water or food was given during the experiment. The animals were hydrated by being placed on a lettuce diet (ad libitum) for 24 h before the start of the experiment.

Experimental design

Controls

Six animals were infused with an isotonic, sterile sodium bicarbonate solution (1.39%) at a rate of 0.3 ml/min for 5 h. One hour after the start of the bicarbonate infusion an injection of 10 mg/kg sulfisoxazole (10 mg/ml) was administered. Heparinized blood samples (10 U heparin/ml) were obtained at various times. Samples of 2.5 ml blood were obtained just before sulfisoxazole was given, and at 0.25, 0.5, 1, 2, 4, 6, 8 and 10 h after the dose. Samples of 0.5 ml were obtained at 0.75, 1.5, 3, 5 and 7 h. The blood was obtained from the ear-vein in the opposite ear where the sulfisoxazole was injected. The samples were centrifuged and the plasma stored frozen (−20°C) until assayed. The urine was collected before the start of the study, and 1, 3, 5, 7, 11 and 24 h after the sulfisoxazole administration. The urine was allowed to drain into a beaker between urine samples, and at the collection time 2 × 5 ml isotonic saline was used to rinse the bladder to assure complete recovery of the drug excreted during the collection interval. The urine pH was measured in the combined sample. We found that the addition of 10 ml isotonic saline to the urine did not affect the measured urine pH. Five hours from the start of the experiment, after the majority of sulfisoxazole was eliminated, the bicarbonate infusion was terminated.

Experimental animals

The remaining 6 animals were studied under conditions identical to the description above with one major difference: a dose of 35 mg/kg of 4-N-acetyl-sulfisoxazole was injected i.v. one hour before the sulfisoxazole injection, and the isotonic bicarbonate infusion solution contained 4-N-acetyl-sulfisoxazole in a concentration to achieve an approximate infusion rate of 0.15 mg/kg/min of 4-N-acetyl-sulfisoxazole for the 5 h of bicarbonate infusion.

An additional 2.5 ml blood sample was taken before the 4-N-acetyl-sulfisoxazole injection and a 0.5 ml sample 0.5 h before the sulfisoxazole injection.

Sulfisoxazole

Sulfisoxazole was randomly tritiated by catalytic exchange in solution (Amersham). The drug was purified by recrystallization in ethanol together with preparative thin-layer chromatography. The potency was checked by a high-pressure liquid chromatographic method (Jung and Øie, 1980) where the eluent was counted by liquid scintillation in 30-s aliquots. The purified [^{3}H]sulfisoxazole was diluted with non-radioactive sulfisoxazole to achieve an activity of approximately 10 nCi/mg.

Protein binding

Seven-hundred μl plasma was dialyzed against an equal volume of phosphate buffer (0.13 M, pH 7.40 at 37°C for 8 h) using a 1 ml Plexiglass dialysis cell (Technilab Instruments, Pequannock, N.J.) and viscose dialysis membrane (VWR, San Francisco, Calif.).

Analysis

One-hundred μl plasma, equilibrium dialysis buffer or urine (or diluted urine) was deproteinized with 200 μl methanol containing 12 mg/l acetylsulfamethoxazole as the internal standard. The samples were vortexed for 10 s and then centrifuged. Twenty μl of clear supernatant was injected onto a C-18 reverse-phase column (Biorad, Berkeley, Calif.). Eluting solvent was 32% methanol in 0.01 M sodium acetate buffer, pH 4.7, with a flow-rate of 1 ml/min. The eluent was monitored at 254 nm (Altex, Berkeley, Calif.). One-min samples containing sulfisoxazole or 4-N-acetyl-sulfisoxazole were collected from the injected urine samples as they left the detector. To each of these samples, 10 ml Aquasol (New England Nuclear, Boston Mass) was added and the sample counted using a liquid scintillation counter (Packard Tricarb 3375, Packard Instruments, Downers Grove, Ill). The concentration of radioactive sulfisoxazole and 4-N-acetyl-sulfisoxazole was determined using the specific activity of the sulfisoxazole injected *.

Fitting of data

All plasma concentration–time data (total as well as unbound concentrations) were fitted either to a one- or a two-compartment pharmacokinetic model using a procedure

* In animals into which only tritiated sulfisoxazole was injected, the ratio of the specific activity of sulfisoxazole recovered in the urine to the specific activity injected was 0.973 ± 0.46 (mean ± S.D.).

(DrugFun (Holford, 1979)) available through the PROPHET computer system (Castleman et al., 1974). Clearances, based upon unbound and total drug concentration were obtained from the dose and area under the plasma concentration–time curves. The renal clearance was obtained from the total amount excreted unchanged in the urine and the area under the plasma concentration–time curve. The metabolic clearance was obtained from the amount of 4-N-acetyl-sulfisoxazole recovered in the urine and the area under the plasma concentration–time curve. The assumption made is that the metabolite is completely excreted unchanged in the urine *. The half-life is the terminal half-life observed. The apparent volume of distribution is obtained from the clearance and half-life when a one-compartment model was used, or by calculating the steady-state volume (Gibaldi and Perrier, 1975) when a two-compartment model was used.

The elimination clearance of the metabolite is determined from the infusion rate and steady-state plasma concentrations of 4-N-acetyl-sulfisoxazole in the experiment group, and from the total amount excreted in the urine and the area under the plasma concentration–time curve of the metabolite for the control group.

RESULTS

The log-averaged plasma concentrations of total and unbound sulfisoxazole and of total 4-N-acetyl-sulfisoxazole from the control animals and the experimental animals are given in Figs. 1 and 2.

Various pharmacokinetic parameters from the individual animals are given in Tables 1 and 2, and the average unbound fractions of sulfisoxazole and 4-N-acetyl-sulfisoxazole in plasma are given in Table 3. Four of 6 control animals and one of the experimental animals demonstrated two-compartment characteristics for the disposition of sulfisoxazole, while the remainder showed a disposition that could not be differentiated from a one-comparment model. The fitting of the data was done by either a one- or a two-compartment model, depending upon the observed data.

The unbound fraction of sulfisoxazole was substantially increased in the presence of 4-N-acetyl-sulfisoxazole ($P < 0.001$) (see Table 3). The binding of 4-N-acetyl-sulfisoxazole was also decreased in the experimental group when the levels were high, ($P < 0.01$); however, the increase was not as pronounced as for sulfisoxazole. No concentration-dependent binding was observed for sulfisoxazole and 4-N-acetyl-sulfisoxazole in the control rabbits. In the experimental animals the unbound fraction of sulfisoxazole and 4-N-acetyl-sulfisoxazole remained constant during the constant infusion of 4-N-acetyl-sulfisoxazole. In the post-infusion phase the binding of 4-N-acetyl-sulfisoxazole was found to be concentration-dependent in all 6 animals ($P < 0.01$). The binding of sulfisoxazole was only determined at one time point in the post-infusion period. In all 6 animals the unbound fraction at this point was lower than during the infusion period.

The total body clearance (Cl), as well as the total renal clearance (Cl_R) is statistically significantly higher in the experimental group than in the control group ($P < 0.001$) (Table 1). The total metabolic clearance was, on the other hand, slightly decreased ($P <$

* 0.962 ± 0.042 (mean ± S.D.) of infused 4-N-acetyl-sulfisoxazole was recovered in the urine.

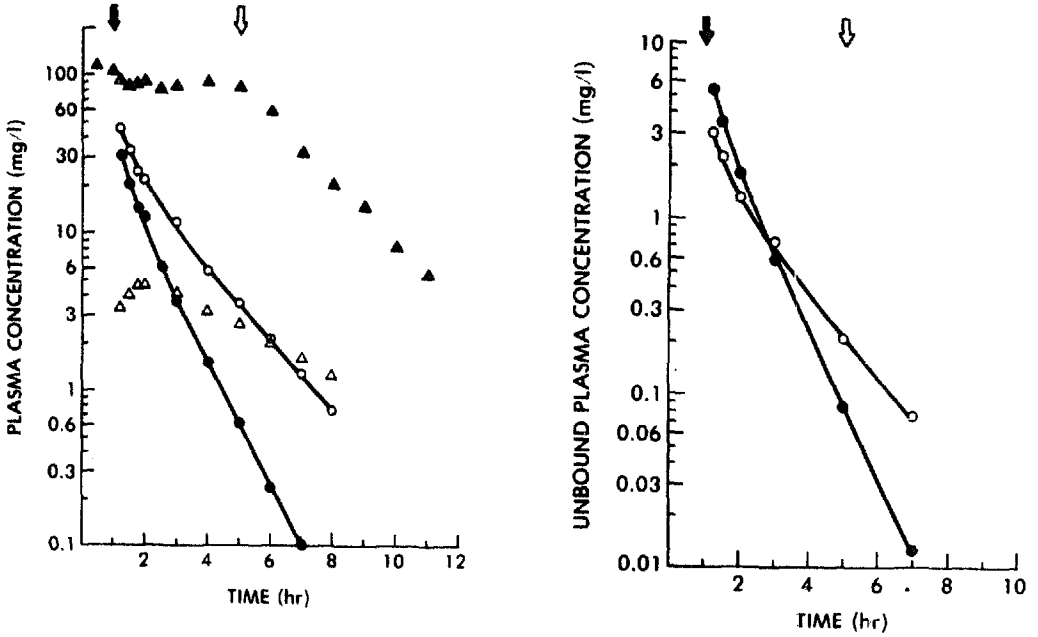

Fig. 1. Log-average plasma concentrations of sulfisoxazole in the absence (○) and presence (●) of infused N-acetyl-sulfisoxazole in 6 rabbits after a 10 mg/kg intravenous dose. (△), Log-averaged plasma 4-N-acetyl-sulfisoxazole concentrations in 6 control animals; and (▲), the log-averaged plasma 4-N-acetyl-sulfisoxazole concentrations in the infused animals. Dark arrow indicates time of injection of sulfisoxazole and open arrow the end of the bicarbonate/4-N-acetyl-sulfisoxazole infusion.

Fig. 2. Log of averaged unbound plasma concentrations of sulfisoxazole in the absence (○) and presence (●) of infused 4-N-acetyl-sulfisoxazole. Dark arrow indicates time of injection of sulfisoxazole and open arrow the end of the bicarbonate/4-N-acetyl-sulfisoxazole infusion. Data from 6 rabbits per experiment.

0.02). In contrast, the total clearance of unbound drug and the renal clearance of unbound drug remained essentially unchanged in the presence of 4-N-acetyl-sulfisoxazole ($P > 0.20$ and $P > 0.80$ respectively). The metabolic clearance of unbound drug, on the other hand, decreased to 1/5 of its control value ($P < 0.001$). The elimination of 4-N-acetyl-sulfisoxazole measured as total clearance, was unaltered, using either unbound or total plasma concentration (see Table 2).

The steady-state apparent volume of distribution is slightly larger in the experimental group in comparison with the control group ($P < 0.02$), while the unbound steady-state apparent volume of distribution is substantially decreased, to half its control value, ($P < 0.001$). The terminal half-life, determined both from the unbound and total plasma concentration data, were lower in the experimental group ($P < 0.01$ and $P < 0.005$ respectively). No statistically significant difference between the terminal half-life from the total and unbound data in the experimental group was observed ($P > 0.05$) while a statistically significant shorter half-life for the unbound concentrations was found in the experimental group ($P < 0.05$). For both groups combined, the half-life determined from the unbound concentrations was lower than the half-life obtained from the total plasma concentrations ($P < 0.005$). The average difference in the half-life, however, was small (4 min).

TABLE 1

PHARMACOKINETIC PARAMETERS [a] FOR SULFISOXAZOLE IN CONTROL AND EXPERIMENTAL ANIMALS

Animal no.	Cl	Cl_R	Cl_m	Cl_u	Cl_{Ru}	Cl_{mu}	V^{ss}	V_u^{ss}	Terminal		Dose [b]
									$t_{1/2}$	$t_{1/2,u}$	
	(ml/min)	(ml/min)	(ml/min)	(ml/min)	(ml/min)	(ml/min)	(l/kg)	(l/kg)	(min)	(min)	(mg/kg)
Control											
1	1.56	1.29	0.26	31.0	25.6	5.0	0.207	4.15	92	92	30.1/2.72
2	2.46	2.26	0.41	36.8	33.7	6.1	0.228	3.16	64	60	28.5/2.56
3	1.43	1.14	0.24	25.6	20.7	4.1	0.224	3.74	109	101	26.8/2.98
4	3.11	2.57	0.43	38.8	32.2	5.4	0.252	3.08	56	55	26.2/2.87
5	1.95	1.55	0.32	37.9	30.2	6.3	0.273	4.65	97	85	24.1/2.56
6	2.89	2.54	0.42	37.2	32.8	6.2	0.309	3.92	74	73	24.0/2.61
Mean	2.24	1.89	0.35	34.6	29.2	5.5	0.249	3.78	82	78	
± S.D.	± 0.70	± 0.64	± 0.08	± 5.2	± 5.1	± 0.9	± 0.037	± 0.60	± 21	± 18	
Experimental											
7	4.11	3.74	0.11	24.8	22.7	0.68	0.281	1.57	47	44	31.9/3.07
8	4.46	4.14	0.32	23.5	21.8	1.62	0.242	1.14	38	34	27.0/2.84
9	5.68	5.60	0.12	42.2	41.6	0.88	0.332	2.33	39	39	29.9/2.76
10	7.18	6.68	0.24	42.5	40.4	1.41	0.351	2.04	34	33	22.4/2.53
11	5.25	5.10	0.31	21.4	20.8	1.27	0.409	1.45	54	47	33.7/4.00
12	3.48	3.56	0.15	22.8	23.3	1.00	0.393	2.22	78	68	26.3/2.10
Mean	5.05	4.80	0.21	29.5	28.4	1.14	0.339	1.79	48	44	
± S.D.	± 1.32 [f]	± 1.21 [f]	± 0.09 [d]	± 10.0	± 9.8	± 0.35 [f]	± 0.064 [d]	± 0.47 [f]	± 16 [e]	± 13 [c]	

[a] Cl is clearance and V is apparent volume of distribution. Subscripts: R, renal; m, metabolic; u, unbound; and ss, steady state.

[b] The ratio denotes the total dose in mg and the weight of the rabbits in kg.

[c] $P < 0.05$.

[d] $P < 0.02$.

[e] $P < 0.01$.

[f] $P < 0.001$.

TABLE 2

TOTAL AND UNBOUND ELIMINATION CLEARANCE OF 4-N-ACETYL-SULFISOXAZOLE

Animal no.	Control		Animal no.	Experimental	
	Cl (ml/min)	Clu (ml/min)		Cl (ml/min)	Clu (ml/min)
1	0.94	18.9	7	1.69	18.7
2	1.47	25.7	8	1.00	7.9
3	0.43	10.0	9	2.08	22.8
4	1.76	22.3	10	1.54	17.0
5	0.91	20.6	11	1.56	26.7
6	1.07	15.6	12	3.44	33.0
$\bar{X}$ ± S.D.	1.10 ± 0.47	18.9 ± 5.5	$\bar{X}$ ± S.D.	1.88 ± 0.84	21.0 [a] ± 8.6

[a] Not statistically significant different from control.

TABLE 3

AVERAGE [a] FREE FRACTION OF SULFISOXAZOLE AND 4-N-ACETYL-SULFISOXAZOLE

Animal no.	Control		Animal no.	Experimental	
	SULF	N-A-SULF		SULF	N-A-SULF
1	0.050	0.050	7	0.166	0.090
2	0.067	0.057	8	0.190	0.127
3	0.056	0.043	9	0.135	0.091
4	0.080	0.079	10	0.169	0.091
5	0.051	0.044	11	0.245	0.058
6	0.078	0.069	12	0.153	0.104
$\bar{X}$ ± S.D.	0.064 ± 0.013	0.057 ± 0.014	$\bar{X}$ ± S.D.	0.176 ± 0.038 [b]	0.094 ± 0.022 [c]

[a] Determined from the ratio of the area under the plasma concentration–time curve for unbound drug to the area under the plasma concentration–time curve for total drug.
[b] Statistically significantly different from control ($P < 0.001$).
[c] Statistically significantly different from control ($P < 0.01$).

In the control group 0.998 ± 0.047 (mean ± S.D.) of the injected dose of sulfisoxazole was recovered in the urine either as sulfisoxazole (0.841 ± 0.047) or 4-N-acetyl-sulfisoxazole (0.158 ± 0.012). In the experimental group 1.006 ± 0.042 of the injected dose was recovered in the urine as sulfisoxazole (0.964 ± 0.039) and 4-N-acetyl-sulfisoxazole (0.042 ± 0.019) *. The infused 4-N-acetyl-sulfisoxazole in the experimental group could be recovered to the extent of 0.962 ± 0.042 in the urine. The ratio of the specific activity

* No metabolite other than 4-N-acetyl-sulfisoxazole was detected.

of sulfisoxazole recovered in the urine to the specific activity of the sulfisoxazole injected in the experimental group was 0.967 ± 0.035 indicating little or no deacetylation of the infused 4-N-acetyl-sulfisoxazole.

The observed urine pH remained, relatively speaking, fairly stable throughout the study and did not differ statistically at any time between the control and experimental group.

DISCUSSION

When 4-N-acetyl-sulfisoxazole accumulates in the rabbit, a large decrease in the ability to acetylate the parent compound is seen as reflected in the metabolic clearance of unbound drug. The total metabolic plasma clearance of sulfisoxazole in the control is 0.35 ml/min per kg body weight which means that the metabolic blood clearance cannot exceed 0.7 ml/min per kg body weight (hematocrit < 0.5). With a hepatic blood flow of approximately 60 ml/min per kg body weight, (Balabaud et al., 1975) sulfisoxazole must be labeled as a low extraction ratio drug (Rowland et al., 1973; Wilkinson and Shand, 1975). For such drugs the metabolic clearance of unbound drug directly represents the intrinsic ability to metabolize the drug, i.e. the capacity and affinity of the enzymes to metabolize the compound. Therefore, 4-N-acetyl-sulfisoxazole serves as an inhibitor of the acetylation of sulfisoxazole (product inhibition). The total metabolic clearance, on the other hand, is decreased to a lesser extent. The total metabolic clearance depends, however, not only upon the intrinsic ability to metabolize the drug, but also on the unbound fraction of drug in plasma for a low extraction ratio compound. Because the unbound fraction increases when 4-N-acetyl-sulfisoxazole is given, the decreased ability to metabolize sulfisoxazole therefore becomes obscured when assessing the metabolism by the total metabolic clearance.

The unbound renal clearance is not different in the experimental group from the control group indicating that the ability to renally eliminate sulfisoxazole in the presence of 4-N-acetyl-sulfisoxazole is unaltered. As the urine pH was not different in the two groups we believe the fraction of drug reabsorbed is unaltered. Because our design precluded an accurate determination of urine flow, certain reservations must be taken with this statement. The high pH should, however, assure a high degree of ionization of sulfisoxazole in the urine, a low ability to reabsorb sulfisoxazole and therefore a relatively low degree of urine-flow sensitivity.

The total clearance of unbound drug, which represents both the ability to renally eliminate and to metabolize the drug, is not statistically significantly different in the two groups. The reason for this is that the metabolism represents only a small part of the overall elimination of sulfisoxazole in the rabbit. Therefore, although the ability to metabolize sulfisoxazole is substantially decreased, the overall unbound clearance is only marginally affected.

The apparent volume of distribution with respect to total plasma concentration is higher in the experimental group, as can be expected from the higher unbound fraction. Based upon the theoretical relationship developed by Øie and Tozer (1979) we would expect an average apparent volume of distribution of 0.559 l/kg in the experimental group based upon the values in the control group, provided cellular binding was unaltered. However, with an observed apparent volume of distribution of 0.339 l/kg we con-

clude that there was a decrease in the cellular binding of sulfisoxazole in the presence of 4-N-acetyl-sulfisoxazole, although not to the same degree as the plasma protein binding. Based upon the given data a 1.96-fold increase in the cellular-unbound fraction versus a 2.76-fold increase in the plasma-unbound fraction occurred. It is not unreasonable to anticipate a decrease in cellular binding because if sulfisoxazole and 4-N-acetyl-sulfisoxazole were to bind and compete for the same sites in plasma, it is possible they would also bind and compete for the same sites cellularly.

Similarly, the decrease in the apparent volume of distribution for unbound drug is larger than would be anticipated from the decrease in plasma protein binding alone.

The urinary pH in rabbits is usually very high, 7.9–8.5. However, in our experience a number of rabbits show a progressive decrease in pH when fasting. To avoid such a decrease in urine during the experiment, which might alter renal reabsorption of sulfisoxazole, an isotonic bicarbonate solution was infused for the first 5 h of our study. In this period, 85–95% of the dose was eliminated from the body. The urine pH was kept high during the whole study by this procedure and at no time was it statistically significantly different from pre-experimental values ($P > 0.05$ at all times).

The recovery of sulfisoxazole and 4-N-acetyl-sulfisoxazole indicate that sulfisoxazole is only eliminated by renal excretion and by metabolism to 4-N-acetyl-sulfisoxazole in the rabbit. It appears that 4-N-acetyl-sulfisoxazole is only renally eliminated, and is not further metabolized nor deacetylated to a detectable degree.

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