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EFFECT OF 4-N-ACETYL-SULFISOXAZOLE ON THE DISPOSITION OF SULFISOXAZOLE IN THE RAT

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SUMMARY

The plasma protein binding of sulfisoxazole in the rat was found to be decreased in the presence of the major metabolite of sulfisoxazole, 4-N-acetyl-sulfisoxazole. This resulted in an increase in the total clearance and apparent volume of distribution of sulfisoxazole. However, both total and renal clearance of *unbound* drug from the body were decreased, indicating that the intrinsic ability to metabolize and renally eliminate sulfisoxazole is decreased by the presence of the major metabolite, 4-N-acetyl-sulfisoxazole.

INTRODUCTION

A metabolite(s) of a drug tends to accumulate in several circumstances: during multiple dosing, particularly if the half-life of the metabolite is long; in patients with renal failure, when the principal route of elimination of the metabolite is renal; and when the elimination is decreased due to drug interactions.

When it is recognized that a metabolite has specific pharmacologic properties of its own, i.e. N-acetyl-procaineamide, an active metabolite of procaineamide, such accumulations are usually taken into consideration in determining the drug regimen. When the metabolite has no pharmacologic activity, the accumulative effect is usually ignored.

Because a metabolite often retains many of the same functional groups as the parent compound, the metabolite could bind to the same proteins in plasma and tissue, to the same metabolic enzymes, and to the same transport molecules in the body as the parent compound, provided that the remaining functional groups are important for these types of binding. If the concentration of the metabolite is sufficiently high, competition for such binding with the parent compound will occur. Alterations in the disposition of the parent compound will subsequently take place through alterations in the apparent volume of distribution, and/or renal or metabolic clearances.

Few reports exist describing the interaction of metabolites with their parent com-

pound, and these reports concern themselves primarily with competition for metabolic enzymes in animals (Jähnchen and Levy, 1972; Ashley and Levy, 1973) and humans (Klotz et al., 1976). One reason for this paucity of reports may well be due to the simultaneous occurrence of several interactions that tend to obscure clear-cut pharmacokinetic interpretations. If, for example, the total clearance of drug from the body is investigated, a decrease in protein binding tends to increase the clearance of a drug that is not blood flow-rate limited in its elimination, when competition for metabolic enzymes or renal secretion tends to decrease the clearance. The net effect may well be that total clearance of drug from the body is not significantly altered. Because the levels of unbound drug will more correctly represent the pharmacologic response, and because only unbound drug is active, clearance of unbound drug, not clearance of total drug (bound and unbound) (total clearance), is the more appropriate parameter. Clearance of unbound drug, in contrast to total clearance, is independent of plasma protein binding when clearance of the drug is not blood flow-rate limited.

To illustrate the possibility of changes in the disposition of a drug by its metabolite and also the differences in interpretation of the data depending on whether unbound clearance was measured, sulfisoxazole (sulphafurazole) was chosen as a model compound. The therapeutic plasma concentration of sulfisoxazole is quite high (50–150 mg/liter), and the plasma concentration of its primary metabolite, 4-N-acetyl-sulfisoxazole, tends to be significant especially in patients with decreased renal function. This latter situation closely parallels the prophylactic administration of sulfisoxazole to renal transplant patients (unpublished results) because both sufisoxazole and 4-N-acetyl-sulfisoxazole are secreted by the acid transport system in the kidneys (Cohen and Pocelinko, 1973).

MATERIALS AND METHODS

Experimental procedure

Studies were carried out in 6 male Sprague–Dawley rats, weighing 238–320 g. The right jugular vein was cannulated under ether anesthesia 5 days before the start of the experiments as described by Upton (1975). The animals were studied in metabolic cages where they were kept for 25 h after each experiment to enable collection of total urine output for a total of 36 h.

A 25 mg/kg dose of sulfisoxazole was injected on two separate occasions in a crossover fashion as follows: (1) 1 h after an infusion of isotonic saline, 0.6 ml/h, which was continued for 10 h; (2) 1 h after a bolus dose of 20 mg/kg of 4-N-acetyl-sulfisoxazole immediately followed by an infusion of 4-N-acetyl-sulfisoxazole, 10 mg/kg/h in an isotonic saline vehicle at a rate of 0.6 ml/h, for 10 h.

Nine heparinized blood samples (0.4 ml) were obtained during each study: at 0 h just before injection of sulfisoxazole, and at 0.5, 1, 2, 3, 5, 7 and 9 h after injection of sulfisoxazole. All samples were withdrawn from the jugular vein catheter. Considerable care was taken to obtain 'main stream' blood samples uncontaminated by the infusion solution. After disconnecting the infusion pump, the remaining infusion solution in the catheter (50–100 μl) was flushed into the bloodstream with 100–200 μl of 0.9% sodium chloride solution containing 20 U of heparin/ml using a syringe attached to a three-way stopcock. The saline solution was then drawn back into the syringe, thereby filling the

catheter with blood. Then, 400 μl of blood was withdrawn into a second syringe containing 8 U heparin (blood sample). Approximately 100 μl of 0.9% sodium chloride solution containing 20 U of heparin/ml was then injected back into the catheter from the first syringe to flush the remaining blood in the catheter into the bloodstream and the infusion was resumed. The whole procedure took less than 2 min. The plasma was removed from the blood samples after centrifugation and the remaining red blood cells were mixed with an equal volume of isotonic saline and reinjected via the jugular vein catheter into the animal to prevent a significant decrease in the hematocrit value during the experiment.

Two days after the crossover experiment, the animals were exsanguinated. Approximately 15 ml of heparinized blood (20 U heparin/ml) was obtained via the jugular vein catheter. The blood was centrifuged at 2500 rpm for 15 min and the plasma used for equilibrium dialysis [1].

Equilibrium dialysis

One-milliliter aliquots of plasma from the exsanguinated animals were individually spiked with concentrated sulfisoxazole and 4-N-acetyl-sulfisoxazole to yield concentrations in the region observed during the crossover experiment. Of the aliquot 900 μl were dialyzed against 0.9 ml of isotonic phosphate buffer (0.13 M), pH 7.4, for 8 h at 37°C in a shaking waterbath using a Visking dialysis membrane and a Metro Scientific Plexiglass 1-ml dialysis cell.

Assays

Rieder's (1972) method for the determination of unchanged and total sulfonamides was used with the following modifications:

Unchanged sulfisoxazole. A 100-μl sample of plasma or urine was added to 1 ml of McIlvain citrate buffer, pH 5.5 (double concentration), and extracted with 2.5 ml of ethyl acetate by shaking for 5 min. The samples were then centrifuged at 2500 rpm for 8 min. The following reagents were added and mixed in the following sequence: 2 ml of the ethyl acetate layer, 0.25 ml of 2 N HCl in acetone (1 ml of 8 N HCl + 3 ml acetone), 0.25 ml 0.1% $NaNO_2$ in water/acetone mixture (1 + 3); 6 min later, 0.25 ml of 5% sulfamic acid in water/acetone mixture (1 + 3) and 3 min later, 0.25 ml of absolute methanol. All reagents used were of analytical grade. The resulting color was read spectrophotometrically at 545 nm.

4-N-acetyl-sulfisoxazole. A 100-μl plasma or urine sample was added to 1 ml of H_2O and mixed. To the diluted plasma, 0.5 ml of 8 N HCl and 0.5 ml 20% trichloroacetic acid was added and thoroughly mixed. The mixture was covered with aluminum foil and placed in a boiling water bath for 90 min. After hydrolysis and cooling, the mixture was neutralized with 0.8 ml of 5 N NaOH, and 2 ml of McIlvain citrate buffer, pH 5.5 (5-fold strength), was added to bring the pH to approximately 5.5 and then extracted with 2.5 ml of ethyl acetate by shaking for 5 min. Two milliliters of the ethyl acetate layer was

[1] The protein binding of sulfisoxazole and 4-N-acetyl-sulfisoxazole was found to be identical in heparinized plasma and in serum, indicating that the heparin did not affect the binding studies.

treated as described earlier. The difference between the total sulfisoxazole concentration and the amount of unchanged sulfisoxazole was considered to represent 4-N-acetyl-sulfisoxazole. Use of specific high pressure liquid chromatography confirmed that more than 95% of the difference between total and unchanged sulfisoxazole processed as described herein is indeed 4-N-acetyl-sulfisoxazole (Jung and Øie, unpublished observations).

All standars used were treated identically to the biologic samples.

Calculation of pharmacokinetic parameters

The individual plasma drug vs time data were fitted to a two-compartment model using the WTFITFUN routine of the PROPHET computer system where the weighing factor was $1/Cp^2$ (Castleman et al., 1974).

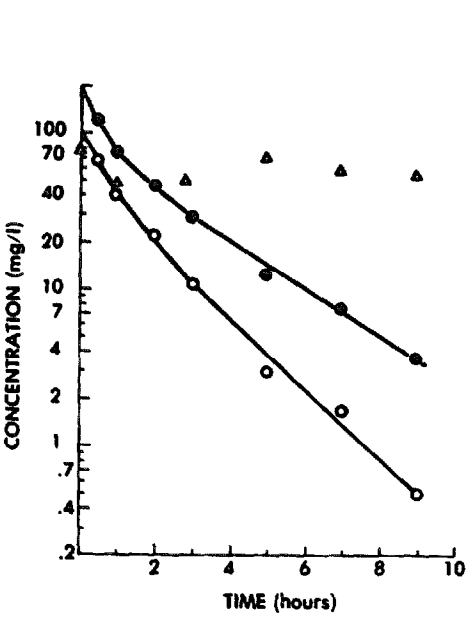
Total and unbound drug clearances were determined by dividing dose by the areas under the plasma unbound and total concentration of drug vs time curves, respectively. The areas under the drug curves were obtained by the trapezoidal rule (Gibaldi and Perrier, 1975). The apparent volumes of distribution were obtained from the total and unbound clearances and the terminal slopes of plasma concentrations of drug vs time curves determined from the computer fit. Renal clearance was determined by multiplying total clearance by the fraction of unchanged drug in the urine in 36 h to total drug administered. All statistical comparisons were carried out by a non-parametric *t*-test (Sign test, Daniel, 1974).

RESULTS

Typical plasma concentrations of total and unbound sulfisoxazole vs time in the absence and presence of 4-N-acetyl-sulfisoxazole are shown in Figs. 1 and 2, respectively. No detectable plasma concentration of 4-N-acetyl-sulfisoxazole was found in the absence of 4-N-acetyl-sulfisoxazole infusion with the assay method used. Of the amount of sulfisoxazole injected 90–95% was recovered from the urine as sulfisoxazole and 4-N-acetyl-sulfisoxazole.

The average two-compartment model parameters obtained, the average clearances of total (bound and unbound) and unbound drug, the average renal clearance, the average fractions unchanged drug excreted, the average apparent volume of distribution of total drug, and the average urinary pH in the absence and presence of 4-N-acetyl-sulfisoxazole are presented in Table 1. The individual clearances of unbound and total sulfisoxazole are presented in Table 2.

The fast and slow rate constants (α and β) were not statistically significantly altered between the two experiments ($0.30 < P < 0.40$). The intercepts (A and B), however, decreased significantly statistically in the presence of 4-N-acetyl-sulfisoxazole ($P < 0.02$), which is consistent with the increase in the apparent volume of distribution ($P < 0.02$) and the increase in the unbound fraction of sulfisoxazole in plasma ($P < 0.02$). Both the total and renal clearances of drug were found to be significantly larger in the presence of 4-N-acetyl-sulfisoxazole ($P < 0.02$) than in the absence of this compound, which is consistent with the increase in the unbound fraction of drug in plasma for a low extraction ratio drug (Wilkinson and Shand, 1975). Clearance of unbound drug, however, was



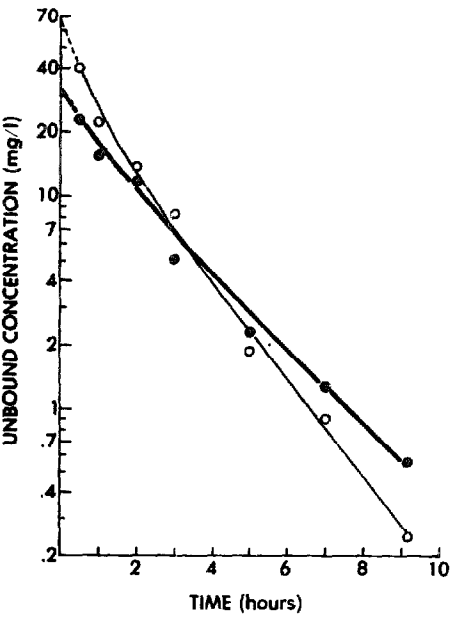


Fig. 1. Plasma concentration of sulfisoxazole (bound and unbound) in the absence (●) and presence (○) of infused 4-N-acetyl-sulfisoxazole (△) in a representative rat. No detectable plasma levels of 4-N-acetyl-sulfisoxazole were found in the absence of infused 4-N-acetyl-sulfisoxazole. Doses: sulfisoxazole, 25 mg/kg; 4-N-acetyl-sulfisoxazole, 20 mg/kg injected 1 h before study followed immediately with a 10 mg/kg/h infusion continued throughout the study.

Fig. 2. Unbound plasma concentration of sulfisoxazole in the absence (●) and presence (○) of infused 4-N-acetyl-sulfisoxazole in the same rat as in Fig. 1. Doses were as described in Fig. 1.

TABLE 1

PHARMACOKINETIC PARAMETERS OF SULFISOXAZOLE IN RATS WITHOUT AND WITH CONCOMITANT INFUSION OF 4-N-ACETYL-SULFISOXAZOLE [a]

Parameter	Without 4-N-acetyl-sulfisoxazole	With 4-N-acetyl-sulfisoxazole
A	93 ± 20 mg/liter	68 ± 30 mg/liter
α	1.78 ± 0.29 h^{-1}	1.89 ± 0.29 h^{-1}
B	101 ± 11 mg/liter	67 ± 10 mg/liter
β	0.39 ± 0.04 h^{-1}	0.40 ± 0.06 h^{-1}
Apparent volume of distribution	211 ± 34 ml/kg	346 ± 71 ml/kg
Total clearance	1.21 ± 0.10 ml/min/kg	2.05 ± 0.27 ml/min/kg
Total renal clearance	0.83 ± 0.08 ml/min/kg	1.38 ± 0.15 ml/min/kg
Unbound clearance of drug	11.8 ± 3.1 ml/min/kg	7.8 ± 1.4 ml/min/kg
Fraction excreted unchanged	0.68 ± 0.02	0.69 ± 0.03
Urine pH	6.4 ± 0.2	6.5 ± 0.2

[a] Average ± standard error of the mean for 6 rats. The 4-N-acetyl-sulfisoxazole concentration was on the average approximately 80 mg/liter.

TABLE 2

INDIVIDUAL CLEARANCES OF TOTAL (BOUND AND UNBOUND) AND UNBOUND SULFISOXAZOLE IN RATS WITHOUT AND WITH CONCOMITANT INFUSION OF 4-N-ACETYL-SULFISOXAZOLE

Rat no.	Total clearance (ml/min/kg)		Clearance of unbound drug (ml/min/kg)	
	Without 4-N-acetyl-sulfisoxazole	With 4-N-acetyl-sulfisoxazole	Without 4-N-acetyl-sulfisoxazole	With 4-N-acetyl-sulfisoxazole
1	1.01	1.57	14.8	10.6
2	1.51	3.19	25.3	12.6
3	1.31	1.78	7.0	5.1
4	0.92	1.53	5.4	4.4
5	1.44	2.54	6.8	5.0
6	1.09	1.71	11.7	9.3
Mean	1.21	2.05	11.8	7.8
S.E.M.	0.10	0.27	3.1	1.4

statistically significantly reduced in the presence of 4-N-acetyl-sulfisoxazole ($P < 0.02$), which should be independent of changes in the plasma protein binding of drug.

When the unbound fraction of sulfisoxazole or the unbound fraction of 4-N-acetyl-sulfisoxazole in plasma was plotted against the total molar plasma concentration of these compounds in the same plasma, identical curves are seen (Fig. 3). When sulfisoxazole

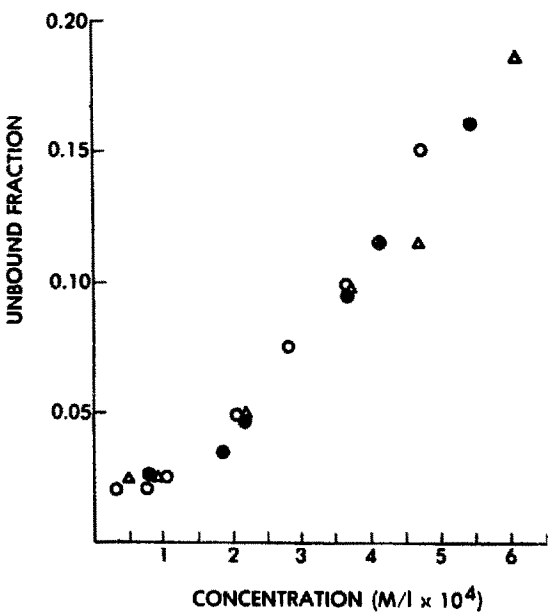


Fig. 3. The unbound fraction of sulfisoxazole in plasma in the absence (○) and presence (●) of exogenous 4-N-acetyl-sulfisoxazole in plasma, and the unbound fraction of 4-N-acetyl-sulfisoxazole in the absence of exogenous sulfisoxazole (△). The abscissa represents the molar concentration of sulfisoxazole and 4-N-acetyl-sulfisoxazole when administered separately and when combined in plasma.

and 4-N-acetyl-sulfisoxazole were mixed in the same plasma and the unbound fractions of sulfisoxazole plotted against the total molar concentration of sulfisoxazole and 4-N-acetyl-sulfisoxazole, the relationship was identical to that seen for sulfisoxazole and 4-N-acetyl-sulfisoxazole separately (Fig. 3), indicating that sulfisoxazole and 4-N-acetyl-sulfisoxazole have identical binding characteristics in rat plasma.

Based on this fact, the unbound fraction of sulfisoxazole was plotted against the total concentration of sulfisoxazole and 4-N-acetyl-sulfisoxazole in the plasma from the individual animals. The unbound fraction of sulfisoxazole during the crossover experiments was estimated from the resultant curves and the estimates were used to calculate the unbound concentration of sulfisoxazole at various times. From the limited number of samples obtained from each animal during equilibrium dialysis, it appeared that in all cases sulfisoxazole and 4-N-acetyl-sulfisoxazole at equal concentrations had the same approximate effect on the unbound fraction of sulfisoxazole.

DISCUSSION

The most important observation in these studies is the decrease in the clearance of unbound sulfisoxazole in the presence of its major metabolite, 4-N-acetyl-sulfisoxazole. For a low extraction ratio drug like sulfisoxazole, the clearance of unbound drug is independent of protein binding and represents only the intrinsic ability of the eliminating organs to eliminate drug (Wilkinson and Shand, 1975). Because the fraction of drug excreted unchanged in the urine was unaltered in the presence and absence of 4-N-acetyl-sulfisoxazole, both renal and metabolic ability to eliminate sulfisoxazole were diminished under the experimental conditions.

The decrease in metabolic elimination is best explained by product inhibition, i.e. competition between sulfisoxazole and 4-N-acetyl-sulfisoxazole for binding to the same enzyme, and indicates that the acetylation of the primary aromatic amine does not change the binding to the acetylating enzyme. In vitro studies of the acetylation of isoniazid support this suggestion that an acetylate metabolite can decrease the acetylation of the parent compound (Weber, 1973). Accumulation of metabolites of sulfisoxazole, e.g. in cases of decreased renal function, might well have a profound effect on the metabolism of sulfisoxazole in man and also reduce elimination of drug by this route, which is indicated in a study by Reidenberg et al. (1969).

The decreasc in the ability of the kidney to eliminate sulfisoxazole is best explained by a reduction in the active secretion of sulfisoxazole. Both 4-N-acetyl-sulfisoxazole and sufisoxazole are actively secreted. A number of actively secreted acids are known to compete for active renal secretion, and the acidic compounds sulfisoxazole and 4-N-acetyl-sulfisoxazole could well behave similarly. Although renal reabsorption of sulfisoxazole is sensitive to the pH of urine (Cohen and Pocelinko, 1973), no changes in reabsorption are expected because no difference in the urine pH between the two experiments was found. Furthermore, because the reabsorption is passive, the presence of the metabolite is not expected to alter the reabsorption of sulfisoxazole. The tubular filtration of unbound sulfisoxazole is directly equal to the glomerular filtration rate. Provided that 4-N-acetyl-sulfisoxazole does not affect the glomerular filtration rate, the change in protein binding should not affect the contribution of glomerular filtration rate to the

renal unbound clearance. However, the clearance of total sulfisoxazole was found to be increased and not decreased despite a reduction in the intrinsic ability to eliminate sulfisoxazole. Sulfisoxazole is a low extraction ratio drug, and the total clearance in contrast to the clearance of unbound drug is linearly dependent on the unbound fraction of drug in plasma. The increase in the unbound fraction of sulfisoxazole due to the presence of 4-N-acetyl-sulfisoxazole is therefore much greater than the decrease in the intrinsic ability to eliminate sulfisoxazole. If one only were to look at the total clearance, it would appear that the presence of 4-N-acetyl-sulfisoxazole increases the ability to eliminate sulfisoxazole. A determination of the half-life (the terminal half-life), on the other hand, would suggest that 4-N-acetyl-sulfisoxazole apparently has little or no effect, because the half-lives are practically identical. However, by proper analysis of these data, the intrinsic ability is found to be decreased.

If we accept the theory that only unbound sulfisoxazole is free to diffuse and to react with receptor sites (Anton, 1960), determination of the concentration of unbound drug in plasma and the clearance of unbound drug is the only procedure for monitoring drug therapy. To use the plasma concentration and clearance of total drug (bound and unbound) may therefore be erroneous and misleading as an accurate indicator of pharmacokinetic changes in vivo.

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REFERENCES

Anton, A.H., The relation between the binding of sulfonamides to albumin and their antibacterial efficacy. J. Pharmacol. Exp. Ther., 129 (1960) 282–290.

Ashley, J.J. and Levy, G., Kinetics of diphenylhydantoin elimination in rats. J. Pharmacokin. Biopharm., 1 (1973) 99–102.

Castleman, P.A., Russell, C.H., Webb, F.N., Hollister, C.A., Siegel, J.R., Zolonik, S.R. and Fram, D.M. The implementation of the Prophet system. Natl. Comput. Conf. Exposition Proc., 43 (1974) 457–468.

Cohen, M. and Pocelinko, R., Renal transport mechanisms for the excretion of sulfisoxazole. J. Pharm. Exp. Ther., 185 (1973) 703–712.

Daniel, W.W., Biostatistics: A Foundation for Analysis in the Health Sciences. John Wiley and Sons Inc., New York, 1974, pp. 333–339.

Gibaldi, M. and Perrier, D., Pharmacokinetics. Marcel Dekker Inc., New York, 1975, p. 293.

Jähnchen, E. and Levy, G., Inhibition of phenylbutazone elimination by its metabolite oxyphenbutazone. Proc. Soc. Exp. Biol. Med., 143 (1972) 963–965.

Klotz, U., Antonin, V.H. and Bieck, P.R., Comparison of the pharmacokinetics of diazepam after single and subchronic doses. Eur. J. Clin. Pharmacol., 10 (1976) 121–126.

Reidenberg, M.M., Kostenbauder, H. and Adams, W.P., Rate of drug metabolism in obese volunteers before and during starvation and in azotemic patients. Metabolism, 18 (1969) 209–213.

Rieder, J., Quantitative determination of the bacteriostatically active fraction of sulfonamides and the sum of their inactive metabolites in the body fluids. Chemother., 17 (1972) 1–21.

Upton, R., Simple and reliable method for serial sampling of blood from rats. J. Pharm. Sci., 64 (1975) 112–114.

Weber, W.W., In Fishman, W.H. (Ed.), Acetylation of Drugs in Metabolic Conjugation and Metabolic Hydrolysis, Vol. III, Academic Press, New York, 1973, pp. 249–296.

Wilkinson, G.R. and Shand, D.G., A physiological approach to hepatic clearance. Clin. Pharmacol. Ther., 18 (1975) 377–390.