Effect of Serum Protein Binding on Sulfisoxazole Distribution, Metabolism, and Excretion in Rats

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Abstract □ This investigation determined the effect of serum protein binding on the kinetics of sulfisoxazole distribution, metabolism, and excretion. Adult rats, whose serum free fraction of sulfisoxazole (at a total concentration of 81 ± 6 μg/ml) was 0.05–0.24, received a rapid intravenous injection of 20 mg/kg. Sulfisoxazole concentrations in plasma declined biexponentially with time. There were pronounced and reproducible interindividual differences in the total, metabolic, and renal sulfisoxazole clearances, each positively correlated with the serum free fraction of sulfisoxazole. The renal sulfisoxazole clearance had a component unaffected by serum protein binding. The apparent central compartment volume increased with an increasing serum free sulfisoxazole fraction, but the latter had no apparent effect on the first exponential term of the biexponential equation describing sulfisoxazole elimination kinetics in rats. Serum protein binding was a major determinant of intersubject differences in sulfisoxazole excretion and biotransformation kinetics.

Keyphrases □ Protein binding, serum—sulfisoxazole, distribution, metabolism, excretion, rats □ Serum proteins—binding, sulfisoxazole, distribution, metabolism, excretion, rats □ Sulfisoxazole—distribution, metabolism, excretion, effect of serum protein binding, rats □ Antibacterials—sulfisoxazole, distribution, metabolism, excretion, effect of serum protein binding

The pharmacokinetic characteristics of drugs can be markedly influenced by serum protein binding (1–3). For drugs with linear, not blood flow rate-limited, elimination kinetics, total clearance is proportional to the serum or plasma free fraction (4–8). Since there are pronounced interindividual differences in the serum protein binding of some drugs even among healthy animals and humans (9–12), serum protein binding can be a major determinant of interindividual differences in drug elimination kinetics.

Previous studies focused on the relationship between the serum free fraction and the pharmacokinetic constants that describe the multiexponential decline of plasma dicumarol (12) and warfarin (4) concentrations in rats after rapid intravenous injection. These studies have now been extended to sulfisoxazole.

The selection of sulfisoxazole was based primarily on the following considerations:

1. The average serum free sulfisoxazole fraction value in rats is one to two orders of magnitude larger than that of warfarin, which, in turn, is one to two orders of magnitude larger than that of dicumarol.
2. Significant interindividual differences in the serum free sulfisoxazole fraction have been demonstrated in rats (13) and humans (9).
3. Sulfisoxazole serum protein binding in humans is affected by their pathophysiologic status (14).
4. Unlike dicumarol and warfarin, which are eliminated almost entirely by biotransformation, sulfisoxazole is eliminated largely by renal excretion.

The investigation was designed to permit assessment not only of total clearance but also of metabolic and renal clearance values individually.

EXPERIMENTAL

Adult male Sprague–Dawley rats[1], 360–475 g, were used. A two-piece silicone rubber–polyethylene cannula was implanted in the right jugular vein under light ether anesthesia (15, 16). One or 2 days later, the rats were placed in individual metabolism cages with food and water freely available. They received a single sulfisoxazole injection, 20 mg/kg iv, through the cannula; blood samples (0.25 ml) were then obtained at 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, and 10.0 hr, using a technique described previously (16).

Plasma was separated and assayed for sulfisoxazole by the Bratton–Marshall method (17). At the end of the experiment, 3 ml of blood was obtained for the determination of the serum free sulfisoxazole fraction by equilibrium dialysis (14) at 37° against pH 7.4 phosphate buffer containing sulfisoxazole, 100 μg/ml. Urine was collected for 24 hr and assayed for sulfisoxazole and its acetylated and conjugated metabolites following selective extraction with and without prior hydrolysis (18). However, rather than performing the Bratton–Marshall reaction in the ethyl acetate phase with solutions of reagents in acetone (18), the ethyl acetate solution was evaporated under nitrogen, the residue was redissolved in 0.5 ml of 3 *N* HCl and 0.2 ml of 30% trichloroacetic acid, and the Bratton–Marshall reaction was carried out in the usual manner. This modification obviated the stability problems encountered previously (18) and resulted in excellent reproducibility.

The sulfisoxazole concentration data for individual animals were fitted to the equation $C_t = Ae^{-\alpha t} + Be^{-\beta t}$ for plasma concentration C_t at time t by nonlinear least-squares regression (19). Convergence was defined as a relative change in the residual sum of squares $<10^{-4}$. Data in all functions were weighted numerically equal. Apparent distribution volumes and total clearance values were determined from the biexponential equation constants (20). Renal and metabolic clearance values were determined from the product of the total clearance and the dose fraction excreted in the urine as sulfisoxazole and as sulfisoxazole metabolite(s), respectively. The product of the total clearance and the dose fraction not recovered in the urine was designated as the clearance of an unknown.

RESULTS

The free sulfisoxazole fraction in the serum of the 13 rats used ranged from 0.052 to 0.235 at an average ± *SD* total concentration of 81 ± 6 μg/ml. Plasma sulfisoxazole concentrations declined biexponentially with time after rapid intravenous injection (Fig. 1). Excellent replication of plasma concentrations was obtained in six rats that received intravenous sulfisoxazole on two occasions, 1 week apart (Fig. 2). The correlations between total clearance values obtained in the two experiments on each of the six rats and between the serum free fraction values were very strong; there was little quantitative difference between values in any one animal (Figs. 3 and 4). An average of ~88% of the dose was recovered in the urine within 24 hr after injection; the 24–48-hr urine (collected from some animals) contained less than 3% of the dose. About 10% of the amount excreted was in the form of metabolite(s) [*i.e.*, substance(s) assayable by the Bratton–Marshall procedure following organic solvent extraction only after acid hydrolysis].

Table I summarizes the pharmacokinetic data obtained from 13 rats in the first experiment. The renal and metabolic clearance values were determined on the basis of the fraction of the dose excreted in the urine as sulfisoxazole and sulfisoxazole metabolites, respectively, during 24 hr after drug injection. The unknown clearance, based on the fraction of the dose not recovered in the urine, is a reflection of possible extrarenal elimination, conversion of the drug to metabolites that are not determinable by the Bratton–Marshall procedure even upon hydrolysis, and (probably largely) technical imperfections such as incomplete urine re-

[1] Blue Spruce Farms, Altamont, N.Y.

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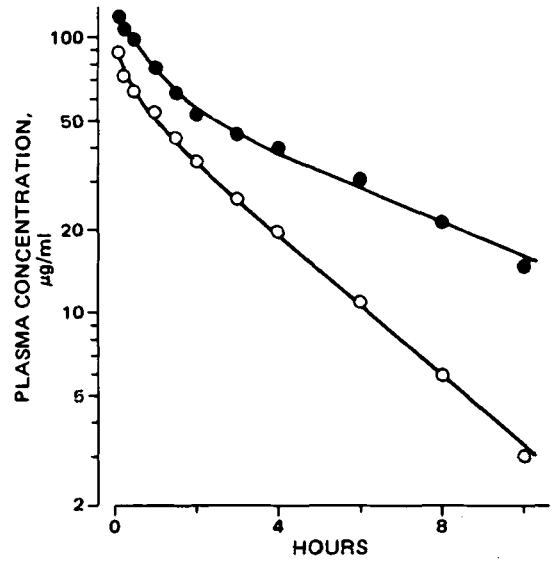

Figure 1—*Time course of plasma sulfisoxazole concentrations of the rat with the lowest serum free fraction (f) value (●, f = 0.052) and the rat with the highest f value (○, f = 0.235) after 20 mg/kg iv. Curves were fitted by nonlinear least-squares regression.*

covery. Consequently, the range of unknown clearance values is wide. More important, there were substantial interindividual differences in the total, renal, and metabolic sulfisoxazole clearances. Therefore, the relationship between these clearance values and the serum free sulfisoxazole fraction in individual animals can be examined.

Figure 5 is a plot of the total sulfisoxazole clearance against the serum free drug fraction. A highly significant correlation was obtained when only data from the first experiment on 13 animals were used for the statistical analysis (a procedure followed also in all subsequent analyses). The total clearance *versus* the free fraction relationship appeared linear but exhibited a substantial positive intercept on the clearance axis. A similarly high correlation and apparently linear relationship were observed between the renal clearance and serum free sulfisoxazole fraction, again with a pronounced positive intercept on the clearance axis (Fig. 6). A highly statistically significant correlation existed between metabolic clearance and serum free fraction, but a plot of these parameters had an intercept close to zero if the animal with the highest metabolic clearance, an apparent outlier, was excluded (Fig. 7). There was no statistically

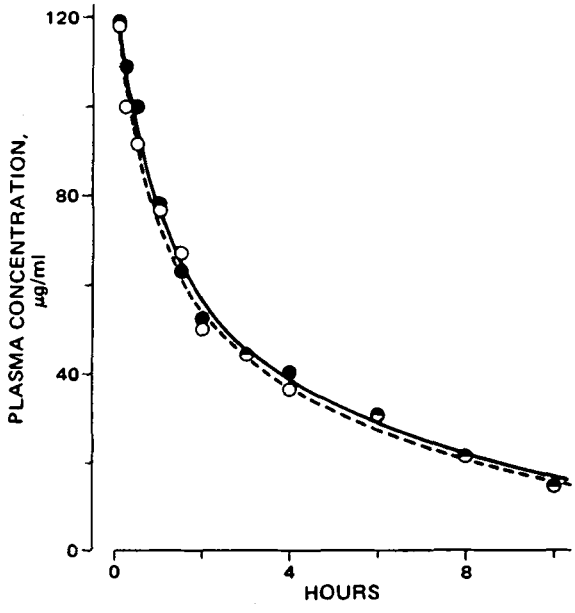

Figure 2—*Time course of plasma sulfisoxazole concentrations of Rat 1 after 20 mg/kg iv in two experiments, 1 week apart. Curves were fitted by nonlinear least-squares regression.*

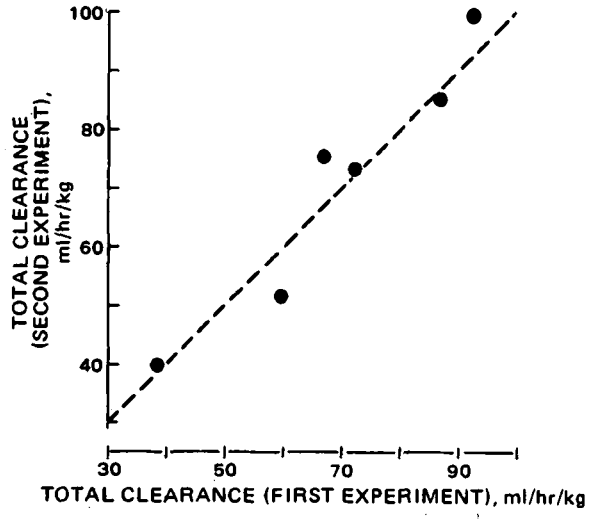

Figure 3—*Relationship between total clearance values for sulfisoxazole determined in two experiments, 1 week apart, in the same six rats. Correlation coefficient equals 0.967, p < 0.005. The broken line in this figure and in several subsequent figures has a slope of unity, indicative of perfect correlation.*

significant correlation between unknown clearance and the serum free sulfisoxazole fraction.

The volume of the apparent central compartment for sulfisoxazole increased significantly with an increasing serum free fraction (Fig. 8, lower panel). A similar trend was evident for the apparent distribution volume (V_{area}), but the relationship was not statistically significant (Fig. 8, upper panel). An examination of the relationships between each biexponential equation constant describing the time course of plasma sulfisoxazole concentrations and the serum free fraction value in individual animals indicated that there was a statistically significant negative correlation between A and the free fraction ($r = -0.666$, $p < 0.02$) but no apparent correlation between B and the free fraction and that there was a statistically very significant positive correlation between β and the free fraction but no apparent correlation between α and the free fraction (Fig. 9).

DISCUSSION

Theoretically, the metabolic clearance of drugs with linear pharmacokinetics should be proportional to their serum free fraction provided that the clearance is much lower than the blood flow rate to the metabolizing organ or tissue (1–3). A linear relationship between *in vivo* total clearance and serum free fraction in rats was demonstrated previously with respect to dicumarol (5), warfarin (1, 4), and bilirubin (6), all of which are eliminated almost entirely by biotransformation.

The hepatic and renal blood flow rates in rats are about 1.7 ml/min/g of liver and 4 ml/min/g of kidneys (21). Based on a liver weight of 30 g/kg

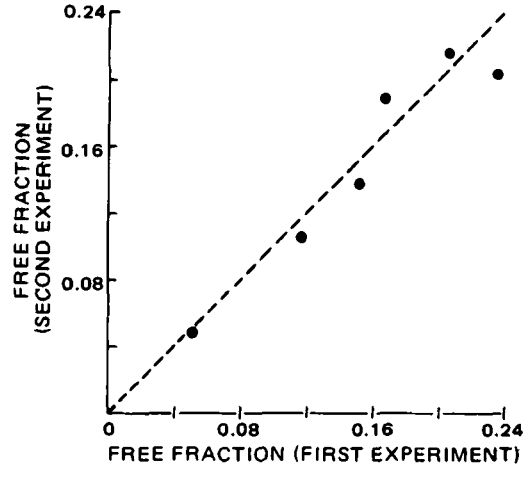

Figure 4—*Relationship between serum free sulfisoxazole fraction determined at the end of two experiments, 1 week apart, in the same six rats. Correlation coefficient equals 0.957, p < 0.005.*

Table I—Sulfisoxazole Pharmacokinetics in Rats

Constant	Mean Value[a]	Range
A, μg/ml[b]	47.4	27.6–61.1
B, μg/ml[b]	65.1	42.8–89.8
α, hr^{-1}	1.08	0.314–1.44
β, hr^{-1}	0.245	0.144–0.313
$t_{1/2}$, hr	2.91	2.21–4.81
V_c, ml/kg	180	149–206
V_{area}, ml/kg	272	215–324
Free fraction in serum	0.144	0.052–0.235
Total clearance, ml/hr/kg	66.5	38.4–92.7
Renal clearance, ml/hr/kg	51.9	27.7–77.9
Metabolic clearance, ml/hr/kg	6.15	2.37–14.1
Unknown clearance[c], ml/hr/kg	8.35	0.742–12.2

[a] $n = 13$. [b] For a 20-mg/kg dose. [c] Total clearance times the fraction of dose not recovered in urine.

of body weight and a kidney weight of 5.7 g/kg of body weight (22), about 3000 ml of blood/hr/kg of body weight flows through the liver, and about 1400 ml of blood/hr/kg of body weight flows through the kidneys. These flow rates are much higher than the total sulfisoxazole clearance observed in this investigation. Consistent with theoretical predictions, the metabolic sulfisoxazole clearance increased proportionately with the serum free drug fraction. Considering the results of this study and of previous investigations on other drugs (4, 5), the proportionality between metabolic clearance and serum free fraction has now been demonstrated at serum free fraction values ranging over three orders of magnitude.

An important discovery in this investigation was the pronounced positive intercept on the clearance axis when renal clearance (and, therefore, also total clearance) was plotted against the serum free fraction. Pending the outcome of studies now in progress, these observations suggest that renal clearance has at least one component that is independent of the serum free sulfisoxazole fraction and at least one other component that is proportional to the serum free fraction. To confirm this preliminary conclusion, it will be necessary to determine the intrinsic and "conventional" renal sulfisoxazole clearances as functions of urine flow rate, urine pH, and drug concentration in plasma so that possible artifacts can be ruled out.

Renal sulfonamide excretion is the net result of glomerular filtration, renal tubular secretion, and partial nonionic back-diffusion from the renal tubular lumen. There is some indirect evidence that the first two of these processes are functions of the plasma free drug concentration and that tubular secretion is describable by Michaelis-Menten kinetics in rabbits (23). Further studies are needed, but this investigation did show that the

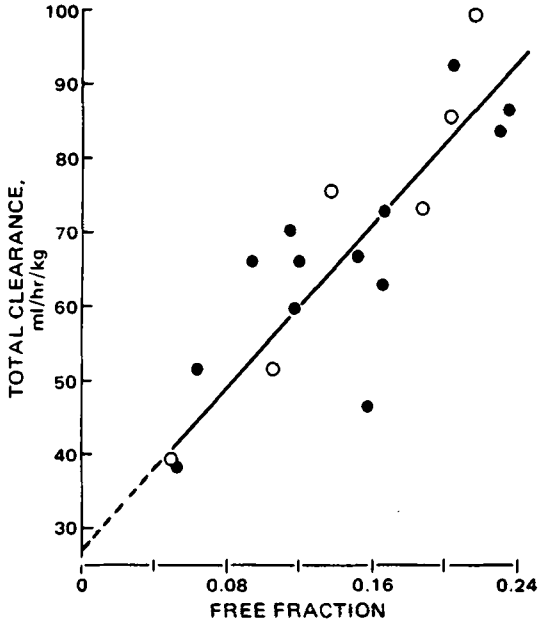

Figure 5—*Relationship between total clearance and serum free sulfisoxazole fraction in 13 rats (●) and in six of these rats retested 1 week later (○). Regression line and correlation coefficient (r) are based on data from first experiment only: r = 0.792, p < 0.005.*

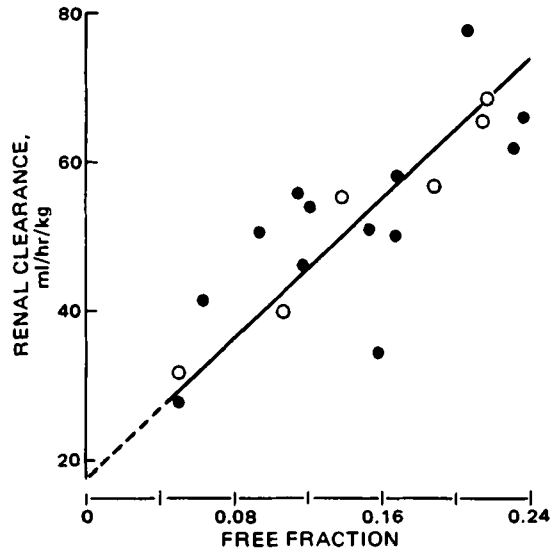

Figure 6—*Relationship between renal clearance and serum free sulfisoxazole fraction. See legend of Fig. 5 for other information; r = 0.732, p < 0.005.*

renal sulfisoxazole clearance increases with increasing serum free fraction.

As previously found with warfarin (4) but not with dicumarol (12), the volume of the apparent central compartment (V_c) for sulfisoxazole increases with an increasing serum free fraction. Therefore, this apparent volume is not the volume of a distinct homogeneous, instantaneously accessible (by intravenous injection) physiologic space such as plasma water. Consistent with this view, the V_c for sulfisoxazole is much larger than the plasma water space, even when extrapolated to a serum free fraction value of zero (Fig. 8).

There is no correlation between α, the exponent of the biexponential equation that characterizes the slope of what is conventionally considered to be the distribution phase of sulfisoxazole elimination, and the serum free sulfisoxazole fraction. A similar lack of correlation was found for dicumarol and warfarin (4, 12). On the other hand, and consistent with previous observations on dicumarol and warfarin (4, 12), there is a strong positive relationship between β and the serum free sulfisoxazole fraction.

Theoretical considerations indicate that β is primarily a function of the free drug fraction in tissues rather than in plasma (24). The weighted

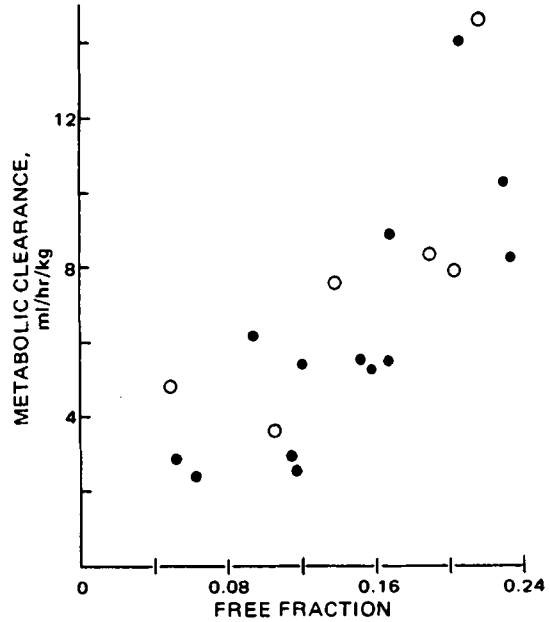

Figure 7—*Relationship between metabolic clearance and serum free sulfisoxazole fraction. See legend of Fig. 5 for other information; r = 0.784, p < 0.005.*

Table II—Serum Protein Binding and Estimated Tissue Binding of Dicumarol, Warfarin, and Sulfisoxazole in Rats[a]

Parameter	Dicumarol	Warfarin	Sulfisoxazole
f_p	0.000497	0.0123	0.144
f_T	0.00258	0.0610	0.399
f_T/f_p	5.19	4.96	2.77

[a] See text for method of tissue binding estimation and sources of data; f_p is the free fraction of drug in plasma or serum, and f_T is the free fraction of drug in tissues.

average apparent free drug fraction in tissues (f_T) can be estimated from the apparent distribution volume (V_{ss}), the free fraction in plasma or serum (f_p), and the real volumes of the body water space (V_w) and the plasma water space (V_p) according to the following relationship (25, 26):

$$V_{ss} = V_p + \frac{f_p}{f_T}(V_w - V_p) \qquad \text{(Eq. 1)}$$

Values of f_T for dicumarol, warfarin, and sulfisoxazole in adult rats were estimated on the basis of average data obtained in this and previous (4, 12) studies, assuming V_w = 700 ml/kg and V_p = 30 ml/kg. These estimates (Table II) indicate substantial binding of all three drugs in tissues. Previously, it was also established that interindividual differences in dicumarol and warfarin f_p are associated with corresponding differences in f_T such that the ratio of f_T to f_p for any one drug is relatively constant over a wide f_p range (27). The β of dicumarol and warfarin increases with increasing f_p, presumably because of the relatively constant relationship between f_p and f_T. The strong positive correlation between sulfisoxazole β and f_p and the inferential indication of significant ($\simeq$ 60%) tissue drug binding suggest that interindividual differences in sulfisoxazole f_p may be associated with corresponding differences in f_T.

The estimated f_T/f_p ratios of dicumarol, warfarin, and sulfisoxazole are quite similar despite large differences in their average f_p values (Table II). The drug f_T/f_p ratio can remain relatively unaffected by compounds that displace the drug from plasma protein binding sites since similar

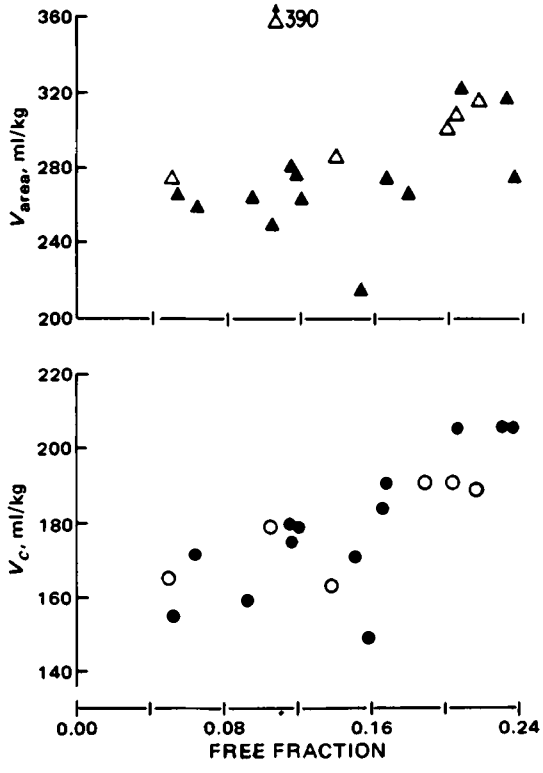

Figure 8—*Relationship between serum free fraction and apparent volume of distribution (V_{area}, upper panel) or apparent volume of the hypothetical central compartment (V_c, lower panel) of sulfisoxazole in rats. Solid symbols are for the first experiment, and open symbols are for the second experiment. Correlation between free fraction and V_c is statistically significant: r = 0.766, p < 0.005. (Correlation between free fraction and V_{area} is not significant: r = 0.454).*

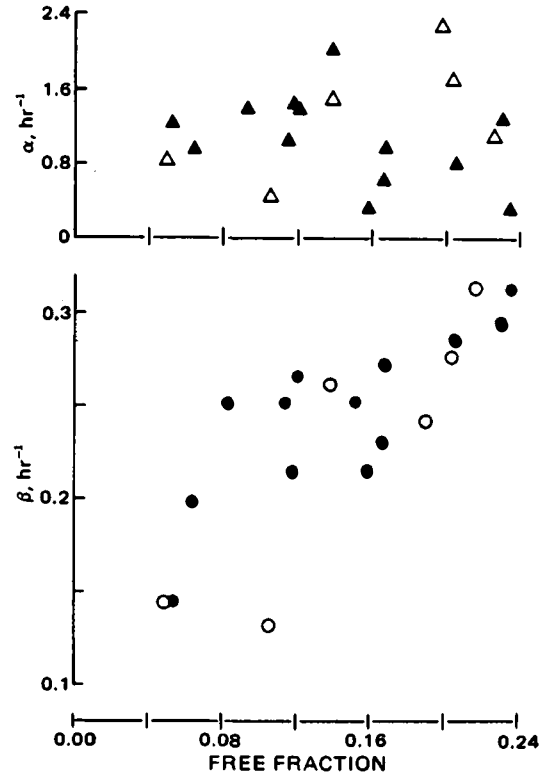

Figure 9—*Relationship between serum free fraction and α (upper panel) or β (lower panel), the exponential constants of the biexponential equation describing the elimination kinetics of sulfisoxazole in rats. Solid symbols are for the first experiment, and open symbols are for the second experiment. Correlation between free fraction and β is statistically significant: r = 0.821, p < 0.001.*

displacement from tissue binding sites may occur (28). This would increase β with little or no change in the apparent distribution volume. The situation is more complicated when drug binding is nonlinear (29).

The serum free sulfisoxazole fraction in 22 healthy adult humans varied from 0.082 to 0.125 at a total concentration of ~9 mg/100 ml (9); it was substantially larger in patients with impaired renal function (14). Serum protein binding may, therefore, be an important determinant of interindividual differences in human sulfisoxazole clearance. The same consideration probably applies to certain other sulfonamides. Consequently, *in vitro–in vivo* correlations, pharmacogenetic characterizations, and similar pharmacokinetic interpretations of the biotransformation of extensively protein-bound sulfonamides should be based on their intrinsic rather than on their total clearance values.

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Analysis of Hydrophobic Amine Antimalarials in Biological Fluids with the Plastic Ion-Selective Electrode

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Abstract □ Plastic ion-selective electrode analysis of the hydrophobic amine antimalarial mefloquine in blood samples was investigated. The direct electrode response in plasma samples provided poor drug sensitivity due to high mefloquine protein binding. The drug was analyzed in whole blood by initial extraction into ether as its trichloroacetate ion-pair. Mefloquine was monitored in whole blood extracts with the electrode to moderately low levels (to 0.4 μg/ml). Rapid blood mefloquine level monitoring by this electrode was demonstrated in a bioavailability study. Mefloquine alkylation with various alkyl halides produced derivatives detectable by the electrode at much lower levels (up to two orders of magnitude) than the parent. A kinetic study of this alkylation reaction revealed that an alkyl amine base was necessary to scavenge the acid produced during reaction and to allow the reaction to go to completion. At room temperature, with benzyl bromide as the reagent, reaction was 99% complete in 30 min and mefloquine could be detected to $\sim 10^{-8}$ *M*, a 100-fold improvement in sensitivity over electrode monitoring of underivatized mefloquine.

Keyphrases □ Electrodes, plastic ion selective—analysis, mefloquine and derivatives in blood, dogs, hydrophobic amines □ Mefloquine—analysis, in blood, plastic ion-selective electrodes, derivatives □ Hydrophobic amines—mefloquine, analysis in blood, plastic ion-selective electrodes □ Antimalarials—mefloquine, analysis in blood, plastic ion-selective electrodes

Recent interest in routine drug level monitoring in biological samples (*e.g.*, blood, plasma, and urine) for determining pharmacokinetic parameters and individualizing dosage regimens had produced a need for analytical methods that can be practically applied in the clinical laboratory. These methods should be rapid, simple, and sensitive, should require minimal sample preparation, and should employ inexpensive instrumentation.

One such method recently was reported (1) from these laboratories for urine methadone analysis. A plastic ion-selective electrode was used that detects hydrophobic cations in aqueous solutions (2, 3). This report describes further studies quantitating hydrophobic amines to low levels in whole blood samples. Amines could be derivatized for detection by the plastic ion-selective electrode, increasing sensitivity up to two orders of magnitude.

The model compounds selected for these studies were the quinolinemethanol antimalarial series mefloquine (I), II, and III, presently under investigation for the treatment of persistent disease caused by *Plasmodia* strains. Problems associated with the blood concentration determination of such hydrophobic amines include glass adsorption, protein binding, and poor sensitivity by conventional detection methods [*e.g.*, high-performance liquid chromatography (HPLC) using UV detection].

EXPERIMENTAL

Materials—Mefloquine, II, and III were used as obtained[1]. The free mefloquine base was precipitated from an alkaline salt solution and recrystallized from water–ethanol to a constant melting point (174–175°). Dodecyl, octyl, and hexyl iodides and tetrabutylammonium bromide were used as obtained from commercial sources. Benzyl bromide was freshly distilled (55–56°/0.03 mm Hg) under nitrogen and stored protected from light under nitrogen until used; at the first sign of a yellow color, fresh reagent was distilled. Methanol and acetonitrile were reagent grade and were distilled in glass prior to use. Electrodes were fabricated as described previously (1).

Fresh whole blood was obtained from beagle dogs and maintained under refrigeration until used, usually within 48 hr. Anticoagulants (usually sodium citrate) were added at collection. Plasma was prepared from whole blood by centrifugation at 5000 rpm for 40 min and collection of the clear supernatant fraction.

Electrodes—Coated wire electrodes were prepared as previously described (1). All electrodes were checked for response to tetrabutylammonium ion before use, and a minimum −56-mv/decade slope (*versus* theoretical −59 mv) was required for electrode acceptability.

Apparatus—All potentiometric measurements were made with a

[1] Through INTERx Research Corp., Lawrence, Kans., from Walter Reed Army Research Institute.

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