Protein Binding and Erythrocyte Partitioning of Disopyramide and Its Monodealkylated Metabolite

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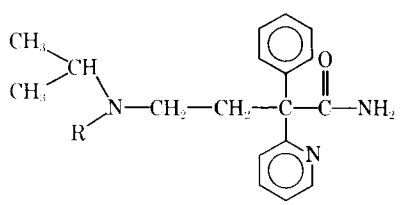
Abstract □ The plasma protein binding of disopyramide, determined by equilibrium dialysis and ultrafiltration, is saturable; the fraction bound to human plasma protein varied from 0.05 to 0.65 with decreasing plasma concentration. The modified Scatchard plots are curved and indicate either the existence of more than one class of independent binding sites or a set of binding sites, where the binding of one molecule of the drug decreases the affinity of another to a previously equivalent binding site. The presence of high concentrations of the monodealkylated metabolite of disopyramide lowered the binding of the parent drug significantly. The fraction of the metabolite bound was less than disopyramide and varied between 0.05 and 0.10. A high concentration of disopyramide decreased the metabolite binding significantly. The red blood cell partitionings of unbound disopyramide (D = 1.2) and the metabolite (D = 1.4) in plasma, studied by centrifugal and ultrafiltration methods, were concentration independent and unaltered by the presence of high concentrations of each other. Equilibration between plasma and red blood cells was quickly obtained for disopyramide but not for the metabolite. Reequilibration of red blood cells containing the drugs gave the same partition coefficients.

Keyphrases □ Disopyramide and monodealkylated metabolite—protein binding and erythrocyte partitioning, equilibrium dialysis and ultrafiltration, Scatchard plots □ Protein binding—disopyramide and monodealkylated metabolite, equilibrium dialysis and ultrafiltration, Scatchard plots □ Erythrocyte partitioning—disopyramide and monodealkylated metabolite

Disopyramide[1] [4-diisopropylamino-2-phenyl-(2-pyridyl)butyramide] (I) is a new and effective antiarrhythmic drug (1–3). It is excreted unchanged and as the monodealkylated metabolite [4-isopropylamino-2-phenyl-(2-pyridyl)butyramide] (II) in humans[2].

The kinetics of drugs in, and their effects on, the body can only be described fully when drug properties in the subcompartments of the central compartment are understood. Evidence exists that the transfer kinetics of drugs are primarily dependent on the concentration of unbound or free, and possibly unionized, drug in plasma water (4). Too frequently, steady-state conditions are assumed within the central compartment without experimental evidence, even though it has been shown that the erythrocytes may act as a "deep compartment" for several drugs, with low rates of transfer with the plasma (5) (Scheme I).



I: R = $(CH_3)_2$—CH
II: R = H

For proper evaluation of studies on the pharmacokinetics and pharmacodynamics of disopyramide[2], it was necessary to delineate the protein binding and red blood cell partitioning of disopyramide and its metabolite separately and in the presence of each other.

This paper compares the methods of equilibrium dialysis and ultrafiltration in the study of protein binding and the methods of centrifugation and ultrafiltration in erythrocyte partitioning. The methods of analysis were spectrofluorometry and liquid scintillation spectrophotometry of labeled drug. It will be shown that disopyramide has unusual binding properties to plasma proteins which, if not considered properly, would lead to the anomalous aberrations of nonlinear pharmacokinetics. These properties suggest a saturable binding to one set of binding sites with high affinity and a less saturable set of binding sites with lower affinity where the fraction bound is a function of the concentration of free drug and/or metabolite in the plasma water.

EXPERIMENTAL[3]

Reagents—Disopyramide phosphate[1] in sterile aqueous solution, ^{14}C-disopyramide phosphate[1], and ^{14}C-monodealkylated metabolite[1] were used.

The radiolabeled disopyramide was synthesized by the method described by Karim *et al.* (6) and had a specific activity of 19.5 μCi/mg. The ^{14}C-labeled monodealkylated metabolite was obtained by isolation from the urine of volunteers who had received the labeled parent drug. Pure metabolite, with a specific activity of 0.16 μCi/mg, was obtained by the method of Karim *et al.* (6).

[1] Norpace is the phosphate salt of disopyramide, G. D. Searle & Co., Skokie, Ill.

[2] P. H. Hinderling and E. R. Garrett, manuscript in preparation.

[3] A Foci model MK-1 spectrofluorometer (Farrand Optical Co., New York, N.Y.) was used in the analysis of disopyramide. A Beckman model LS 100 scintillation counter (Beckman Instruments, Fullerton, Calif.), efficiency determined by an external standard method, and a Packard Tri-Carb 3003 scintillation counter (Packard Instruments Co., Downers Grove, Ill.), efficiency determined by the channel ratio method, were used for the measurement of labeled disopyramide and the monodealkylated metabolite. The pKa′ was determined with a recording titrator, type TTT 1c/SBR^{2}c (Radiometer, Copenhagen, Denmark) equipped with a combination electrode (Corning, New York, N.Y.). Centriflo membrane ultrafilters with conical supports and tubes (Amicon Corp., Lexington, Mass.) were used for ultrafiltration; Visking membranes (Visking Corp.) were used for equilibrium dialysis. A model MB microhematocrit centrifuge (International Equipment Co., Needham Heights, Mass.), tubes (Yankee, Clay Adams, Division of Becton, Dickinson and Co., Parsippany, N.J.), and a microcapillary reader (International Equipment Co., Needham Heights, Mass.) were used to determine the hematocrit.

The liquid scintillation reagent used was Aquasol, New England Nuclear, Boston, Mass. *tert*-Butyl hydroperoxide was obtained from Matheson, Coleman and Bell, Norwood, Ohio.

Heparinized blood and human plasma[4] with known protein fractions were obtained from healthy volunteers who had no prior drug intake. All organic solvents used were of analytical reagent grade.

pKa' Determination of Disopyramide—The differential potentiometric method of Parke and Davis (8) was applied to the titration of 10.0 ml of a sterile aqueous disopyramide solution (3.0 mg/ml = 6.86×10^{-3} *M*) in 0.0045 *N* HCl with 0.0050 *N* NaOH. A solution of 10.0 ml of 0.0045 *N* HCl was titrated with 0.0050 *N* NaOH as the blank.

Analytical Procedures—The spectrofluorometric assay described by Ranney *et al.* (9) was used. This method depends on the fluorescence exhibited by disopyramide in strong acid solution at 410 nm, activated at 275 nm. The original procedure was modified in the measurement of plasma drug concentration to precipitate the plasma proteins by trichloroacetic acid. Plasma (3.0 ml) and water (0.5 ml) were mixed in a centrifuge tube, which was shaken and centrifuged for 2 min. Neutralization was effected by the addition of 1.5 ml of 2.5 *N* NaOH. An aliquot (2.0 ml) of the supernatant solution was carried through the methylene chloride (10.0 ml) extraction procedure. Separate calibration curves were prepared for drug in phosphate buffer solutions and/or plasma in a concentration range between 5×10^{-6} and 2.74×10^{-5} *M* disopyramide. This precipitation of plasma proteins decreased the background significantly.

The liquid scintillation spectrophotometry for ^{14}C-disopyramide and its ^{14}C-metabolite used duplicate aliquots of 100–500 μl of plasma or plasma water pipeted into liquid scintillation vials containing 10 ml of a scintillation reagent. The samples were measured for 10 or 20 min, and the measurements were repeated four times. Corrections were made for background and efficiency. The efficiency was determined either by using an external standard or by the channel ratio method (10), depending on the instrument used.

Plasma water aliquots with slight degrees of hemolysis were subjected to a bleaching procedure prior to measurement to minimize color quenching. Ten to 25 μl of 1 *N* NaOH was added to the plasma water aliquots placed in liquid scintillation vials. The vials were then kept at 37° for 2 hr and for an additional 8 hr after the addition of 10 μl of *tert*-butyl hydroperoxide. Subsequently, 10 ml of a scintillation reagent was added to each vial and the counts were measured. Corrections were made for background and efficiency.

Protein Binding of Disopyramide and Its Monodealkylated Metabolite—Equilibrium dialysis and ultrafiltration were used to study the protein binding of disopyramide in human plasma. A three-chamber dialysis apparatus (11) was used for equilibrium dialysis. The chambers were separated by two membranes. Plasma was placed in the middle chamber, disopyramide in sterile pH 7.4 isotonic phosphate buffer (5.50×10^{-5}–1.10×10^{-3} *M*) was placed in one outer chamber, and sterile buffer was placed in the other. The apparatus had been sterilized previously by steam in an autoclave, except for the membranes and O-rings which were sterilized under UV light. Dialysis was effected for 48 hr at 4° and for 16 hr at 37 and 25°, respectively.

The achievement of equilibrium was ascertained by the equivalence of disopyramide concentrations in both outer chambers. Disopyramide was assayed by the spectrofluorometric method in both outer chambers and in the middle chamber. A pH of 7.15–7.45 was maintained in all three chambers and was determined at the beginning and end of the experiment. The total drug concentration in plasma (middle chamber) at equilibrium ranged between 2.89×10^{-4} and 1.87×10^{-6} *M*. Preliminary experiments showed no significant membrane binding of disopyramide.

In the ultrafiltration studies, spiked plasma aliquots were half-filtered through high flux cone membranes. Half-filtration was preferred, because complete filtration of plasma with precipitation in the cone from a substrate containing plasma might tend to trap and inhibit the free passage of other soluble plasma components (12–14). Total drug concentrations (bound and unbound) in spiked plasma prior to filtration and the free drug concentration in the generated filtrate were determined either by liquid scintillation spectrophotometry or by spectrofluorometry. The protein binding of disopyramide was studied in the concentration range of 4.25×10^{-4}–5.66×10^{-6} *M*. It can be assumed that the plasma filtrates are equivalent to plasma water; the absence of protein was ascertained in the plasma filtrates (15).

The experiments were conducted at 25°, and the pH was kept within 7.30–7.45 unless otherwise specified. The reproducibility of the results was ascertained by the simultaneous ultrafiltration of each concentration of the drug through two to four different cone membranes.

Preliminary experiments indicated a membrane binding of disopyramide. Two different procedures were used to quantify and overcome this problem.

Procedure A—Plasma water was generated by filtering plasma through high flux cone membranes. Aliquots of plasma water were spiked with drug in the concentration range of 4.25×10^{-4}–3.68×10^{-7} *M* and were half-filtered to quantify the membrane binding as a function of the drug concentration. An apparent concentration-dependent membrane binding ranging from 25 to 45% was found. The membrane binding was significantly decreased to 5.4% (±4.8%) when this procedure was repeated with the same cone membranes filled with identically spiked fresh plasma water aliquots. These findings indicated that the membrane binding was saturable. All membranes used in the definitive protein binding experiments were presaturated routinely with drug by half-filtrations of plasma water spiked with an appropriate drug concentration. Half-filtrations of plasma and plasma water were reached after centrifugations of 18–20 and 3–4 min, respectively, at 1000 rpm.

Procedure B—The magnitude of the membrane binding of disopyramide was estimated from a calibration curve obtained when disopyramide-spiked pH 7.4 phosphate buffer solutions (5.00×10^{-6}–2.76×10^{-3} *M*) were filtered through high flux membranes at 2000 rpm for 5 min. These definitive protein binding experiments were performed without presaturation of the membranes. The influence of pH on binding properties of human plasma was the specific goal in this part of the study. Human plasma was adjusted to pH 7.4, 8.0, and 6.7 prior to filtration.

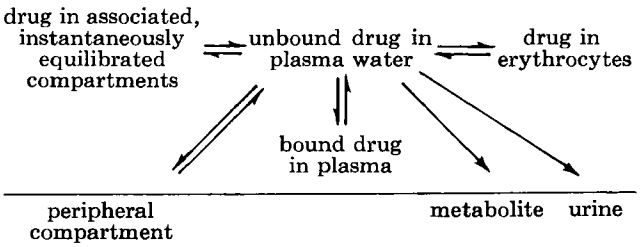
Protein Binding of Disopyramide in Presence of Its Monodealkylated Metabolite in Human Plasma—The ultrafiltration procedure was applied. The protein binding of the ^{14}C-labeled parent drug in the presence of its unlabeled metabolite was investigated at seven different molar concentration ratios of the parent drug to its metabolite, ranging from 9.63 to 0.0045. The concentrations of the labeled parent drug were determined by liquid scintillation spectrophotometry in plasma and plasma water. The extent of the membrane binding of the parent drug in the presence of the metabolite was determined separately at each concentration ratio. The membranes used in the definitive protein binding experiments were saturated by half-filtration of plasma water spiked with an appropriate concentration of both the parent drug and the metabolite.

Protein Binding of the Monodealkylated Metabolite in Human Plasma—The ultrafiltration procedure was used. A significant and apparently concentration-independent membrane binding was found for the metabolite in the magnitude of 20.4% (±3.7%) when spiked plasma water aliquots (2.71×10^{-4}–1.18×10^{-5} *M*) were half-filtered through the membranes. Repeated filtrations of identically spiked plasma water aliquots through the same cone membranes reduced the membrane binding of the metabolite to 1.9% (±1.9%). The membranes used in the definitive experiments were presaturated by one half-filtration of plasma water spiked with appropriate metabolite concentrations.

Protein Binding of the Monodealkylated Metabolite in Presence of Disopyramide in Human Plasma—The ultrafiltration procedure was applied. The protein binding of labeled metabolite in the presence of the unlabeled parent drug was investigated at one concentration ratio: 0.080 of the metabolite to the parent drug. The concentrations of the labeled metabolite in plasma and plasma water were determined by liquid scintillation spectrophotometry. The membranes used in the definitive protein binding experiments were presaturated with appropriate concentrations of the metabolite and the parent drug.

Red Cell–Plasma Water Partition Coefficient of Disopyramide and Its Monodealkylated Metabolite—The determination of the red blood cell partitioning of disopyramide was done in whole blood and red blood cell suspensions in both plasma water and pH 7.4 phosphate buffer. The red blood cell suspensions were prepared from fresh red blood cells separated from plasma by cen-

[4] Blood Bank, Shands Teaching Hospital and Clinics, Gainesville, Fla.



Scheme I—Drug in central compartment

trifugation and washed twice with sterile isotonic sodium chloride solution. After another centrifugation, they were suspended in either buffer or plasma water; the latter was obtained by filtering the separated plasma through the high flux membranes.

Whole blood and red blood cell suspensions were spiked with disopyramide to yield total drug concentrations in the range of 5.50×10^{-5}–8.23×10^{-7} *M*. The hematocrit was routinely determined on the solutions using a microcentrifuge with capillary tubes. The drug concentrations were measured in plasma, plasma water, or buffer by spectrofluorometry or liquid scintillation spectrophotometry after the red blood cells were separated by centrifugation or ultrafiltration through high flux cone membranes. The experiments were carried out at 25°, and the pH was kept between 7.30 and 7.50 and between 7.00 and 7.85 in the red blood cell suspensions and whole blood, respectively.

The red blood cell partitioning of disopyramide as a function of various concentrations of the drug was studied by spiking whole blood and red blood cell buffer suspensions with drug to yield total drug concentrations between 5.50×10^{-5} and 8.23×10^{-7} *M*. Equilibration was allowed for 1 hr. The samples were shaken frequently and subsequently centrifuged for 1 hr for half-filtration. The drug concentrations were measured in plasma, plasma water, and buffer by spectrofluorometry.

The red blood cell partitioning of disopyramide was studied as a function of time by spiking the red blood cell–plasma water suspensions with labeled drug to obtain three different total drug concentrations: 1.58×10^{-4}, 8.23×10^{-5}, and 8.23×10^{-6} *M*. After drug addition, the samples were shaken immediately for 2 min and later at regular time intervals. Aliquots were taken and centrifuged, and the plasma water separated at 5, 6, 9, 18, 33, and 63 min after spiking. The drug concentrations in plasma water were measured by liquid scintillation spectrophotometry. The hematocrit determinations were made on the residual suspension after each aliquot was taken. It was assumed that the obtained hematocrit values reflected those in the removed aliquots. This assumption was confirmed by the consistency among the determined hematocrit values, indicating that the samples were thoroughly mixed at any given time during the experiments. The hematocrit value was determined and the obtained values were corrected by a factor of 0.98 to account for trapped plasma (16).

The repartitioning of disopyramide in the red blood cells into plasma water as a function of time was studied by adding aliquots of the concentrated red blood cells with known hematocrit, obtained from the disopyramide–red blood cell partitioning experiments, to fresh plasma water. The new red blood cell–plasma water suspensions were shaken for 2 min and later at regular time intervals. Samples were taken and centrifuged, and the plasma water separated at 5 and 9 min after spiking. Hematocrit determinations were made on the residual suspensions. The drug concentrations in plasma water were determined by liquid scintillation spectrophotometry.

The red blood cell partitioning of disopyramide in the presence of the monodealkylated metabolite was studied by spiking red blood cell–plasma water suspensions with labeled disopyramide and unlabeled metabolite to yield molar concentration ratios of 0.35 and 0.17 of the parent drug to the metabolite. Aliquots were taken and centrifuged, and the plasma water separated at 5, 9, 33, and 63 min after spiking with the drug. The concentration of the labeled parent drug in plasma water was measured by liquid scintillation spectrophotometry.

The partitioning of the monodealkylated metabolite between red blood cells and plasma water was studied by spiking red blood cell–plasma water suspensions with the labeled metabolite. The plasma water aliquots were separated by centrifugation, and the

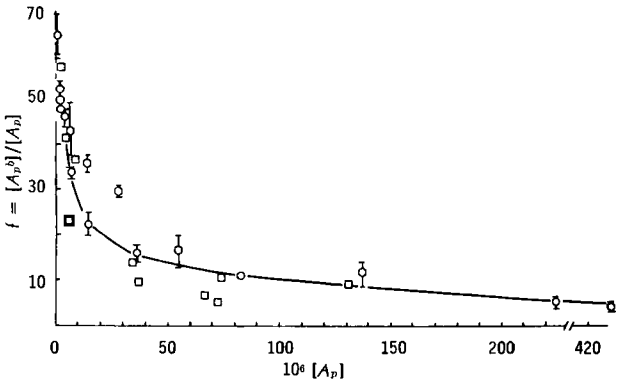


Figure 1—*Plots of the fraction,* f, *of the total drug in plasma that is bound to protein,* $[A_p^b]/[A_p]$, *against the total concentration of drug in the plasma,* $[A_p]$, *for disopyramide as obtained by equilibrium dialysis* (□) *and ultrafiltration through pretreated* (○) *and untreated* (○) *membranes. The standard deviations of the ultrafiltration data are represented by vertical lines through the points. The curve drawn through the points was calculated from an equation given by Hart (20):*

$$f = [A_p^b]/[A_p] = ([A_p^b]/[A_p^u])(1 - f) = \left[\frac{n_1P_1K_1}{K_1[A_p^u] + 1} + \frac{n_2P_2K_2}{K_2[A_p^u] + 1}\right] \times (1 - f)$$

The binding parameters n_1P_1, K_1, n_2P_2, *and* K_2 *were obtained by graphical analysis, where* $[A_p^u]$ *is the concentration of unbound drug in the plasma.*

metabolite concentrations were measured by liquid scintillation spectrophotometry. The procedures and conditions regarding pH, temperature, and hematocrit measurements were the same as for the experiments with the parent drug.

The red blood cell–plasma water partitioning of the monodealkylated metabolite as a function of different concentrations was studied by spiking red blood cell–plasma water suspensions with the metabolite to yield the following concentrations: 1.20×10^{-5}, 8.00×10^{-6}, and 4.00×10^{-6} *M*. The red blood cell–plasma water partitioning of the metabolite as a function of time was studied in the suspensions containing these three concentrations. Aliquots were taken, centrifuged, and separated at 4, 5, 6, 7, 8, 9, 15, 36, and 66 min after spiking.

The repartitioning of the metabolite in the red blood cells into plasma water was studied by adding aliquots of the concentrated red blood cells with known hematocrit, obtained from the metabolite red blood cell partitioning experiments, to fresh plasma water. The new red blood cell–plasma water suspensions were shaken for 2 min and later at regular time intervals. A sample was taken and centrifuged, and the plasma water separated at 66 min after spiking. The red blood cell–plasma water partitioning of the metabolite in the presence of disopyramide was studied in red blood cell–plasma water suspensions spiked with the labeled metabolite and unlabeled parent drug in a molar concentration ratio of 0.86 of the metabolite to the parent drug. Aliquots were taken and centrifuged, and the plasma water separated at 8, 36, and 66 min after spiking.

RESULTS AND DISCUSSION

Protein Binding of Disopyramide—The interaction between a ligand and protein can be formulated as (17):

$$n[A_p^u] + [P] \overset{K}{\rightleftharpoons} [A_nP]$$

Scheme II

where $[A_p^u]$ is the concentration of free or unbound drug in plasma, K is the association constant for the binding of a molecule of drug to one of the equivalent n binding sites of the protein molecule, and $[P]$ is the concentration of protein. Then:

$$[A_p^u] + [A_p^b] = [A_p] \qquad \text{(Eq. 1)}$$

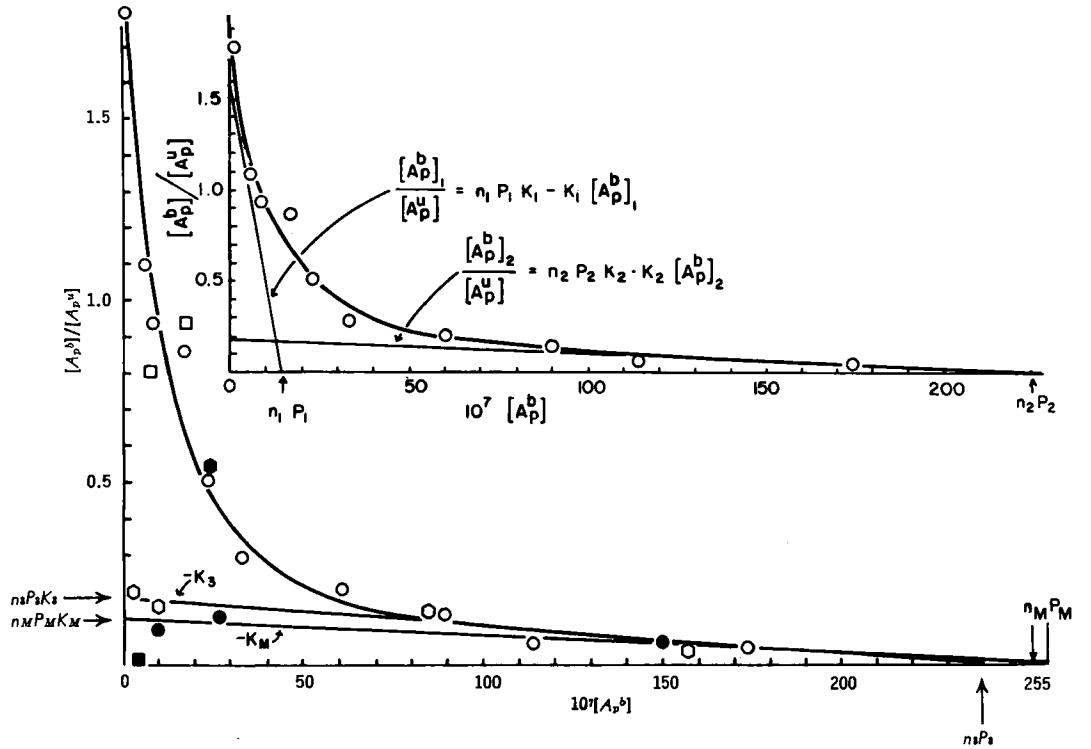


Figure 2—*Modified Scatchard plots for disopyramide and the monodealkylated metabolite in the absence and presence of each other. Key:* ○, *disopyramide alone* ($4.25 \times 10^{-4} - 3.68 \times 10^{-7}$ M); *disopyramide in the presence of different concentrations of metabolite such as* (○) 5.08×10^{-4} M, (●) 2.54×10^{-6} M, *and* (□) 5.08×10^{-7} M; ●, *metabolite alone* ($2.71 \times 10^{-4} - 1.18 \times 10^{-5}$ M); *and* ■, *metabolite* (2.36×10^{-5} M) *in the presence of* 2.97×10^{-4} M *disopyramide. The inset is a modified Scatchard plot of disopyramide with an example of the graphical analysis according to Rosenthal (18) on the premise of the existence of two independent classes of binding sites. Two straight lines,* $[A_p{}^b]_1/[A_p{}^u] = n_1K_1P_1 - K_1[A_p{}^b]$ *and* $[A_p{}^b]_2/[A_p{}^u] = n_2K_2P_2 - K_2[A_p{}^b]_2$, *with the respective slopes of* $-K_1$ *and* $-K_2$, *the respective intercepts on the abscissa of* n_1P_1 *and* n_2P_2, *and the respective intercepts on the ordinate of* $n_1P_1K_1$ *and* $n_2K_2P_2$, *were obtained. The determined binding parameters for disopyramide alone were* $K_1 = 1.00 \times 10^6$/M, $n_1P_1 = 1.60 \times 10^{-6}$ M, $K_2 = 8.44 \times 10^3$/M, *and* $n_2P_2 = 2.25 \times 10^{-5}$ M. *The determined binding parameters for the monodealkylated metabolite alone were* $K_m = 5.39 \times 10^3$/M, *and* $n_mP_m = 2.55 \times 10^{-5}$ M. *For disopyramide in the presence of a high concentration of metabolite* (3.80×10^{-4} M), *they were* $K_3 = 7.98 \times 10^3$/M *and* $n_3P_3 = 2.38 \times 10^{-5}$ M.

where $[A_p{}^b]$ is the concentration of drug bound to protein, and $[A_p]$ is the total drug concentration (bound and unbound) in plasma.

The Langmuir isotherm describes the interaction between small molecules and protein for any given concentration ratio (14) and any number of classes of binding sites:

$$r = \sum_1^i \frac{n_i K_i [A_p{}^u]}{1 + K_i [A_p{}^u]} \qquad \text{(Eq. 2)}$$

where r is the ratio $[A_p{}^b]/[P]$ of the molar concentration of drug bound to protein and the total molar concentration of protein, n_i is number of binding sites in the ith class, and K_i is the association constant for the binding of drug in the ith class. If r, the moles of drug bound per mole of protein, is only proportional to drug concentration, this implies that $K_i[A_p{}^u] \ll 1$ in Eq. 2. Thus, the numbers of binding sites of low affinity are in great excess, so the fraction of the drug bound to protein does not significantly vary with drug concentration and the process appears nonsaturable.

Equation 2 for an ith class of binding sites may be arranged to:

$$r/[A_p{}^u] = n_iK_i - K_ir \qquad \text{(Eq. 3)}$$

and the Scatchard plot (14) of $r/[A_p{}^u]$ against r permits the estimation of n_i and K_i from the slope and intercept. If the nature and/or concentration of the protein is not known, Eq. 3 can be modified (18) to:

$$[A_p{}^b]_i/[A_p{}^u] = n_iK_iP_i - K_i[A_p{}^b]_i \qquad \text{(Eq. 4)}$$

where a plot of $[A_p{}^b]/[A_p{}^u]$ against $[A_p{}^b]$ permits the estimation of K_i and n_iP_i from the slope and intercept. Linear Scatchard or modified Scatchard plots indicate one class of independent binding sites. Curvature in these plots, however, may indicate that there is more than one class of binding sites with different association constants and/or that a class of binding sites exists where the binding of one molecule of the drug affects the affinity of another to a previously equivalent binding site. The fraction of drug bound to a class of independent binding sites can be expressed as:

$$f = \frac{[A_p{}^b]}{[A_p]} = 1/(1 + [A_p{}^u]/n_i[P_i] + 1/n_iK_i[P_i]) \qquad \text{(Eq. 5)}$$

and if the process is nonsaturable, so that the fraction bound is invariant with the concentration of unbound drug, $[A_p{}^u]/n[P_i]$ must be negligible relative to the other two factors in the denominator.

Plots of the fraction of drug bound to plasma protein, f, against the concentration of total drug in the plasma, $[A_p]$, are given in Fig. 1 for the data obtained by equilibrium dialysis and ultrafiltration experiments.

There is good agreement between the data obtained by equilibrium dialysis and ultrafiltration through presaturated cone membranes. When ultrafiltration studies were conducted with untreated membranes, consistently higher values were obtained for f, even when corrections were made for the binding of drug from filtered phosphate buffer solutions. The use of phosphate buffer solutions of drug rather than plasma water solutions leads to an underestimation of the drug fraction bound to the membrane, which results in an overestimation of the fraction bound to plasma protein. It can be argued that phosphate may compete with the parent drug for "binding sites" on the membrane as has been demonstrated to occur between phosphate and small molecules for albumin (19).

The fraction bound to plasma, f, is clearly concentration dependent and increased from 0.05 to 0.65 as $[A_p{}^u]$ decreased from 2.13×10^{-4} to 1.31×10^{-7} M (Fig. 1), indicating saturation of the available binding sites.

The modified Scatchard plot (Fig. 2) for disopyramide is not linear and implies at least two classes of independent binding sites with different affinities or at least one class of binding sites, where

Table I—Influence of Plasma pH on Protein Binding of Disopyramide[a]

Disopyramide Concentration	Fraction of Disopyramide Bound		
	pH 7.5	pH 8.0	pH 6.7
2.70×10^{-4}	0.12, 0.07	0.10	0.08
1.30×10^{-4}	0.10, 0.10	0.15	0.07
5.50×10^{-5}	0.20, 0.13	0.18	0.05
2.74×10^{-5}	0.31, 0.28	0.30	0.15
1.37×10^{-5}	0.35, 0.37	0.36	0.15
5.50×10^{-6}	0.49	0.42	0.29
2.70×10^{-6}	—	0.50	0.12

[a] Ultrafiltrations were performed through untreated membranes.

the binding of one molecule of the drug decreases the affinity of another to a previous equivalent binding site. Graphical and algebraic methods are available (18, 20) to analyze the binding data in accordance with the former hypothesis. A graphical analysis of the data obtained by ultrafiltration with presaturated membranes is shown in the inset of Fig. 2, with the assumption of two independent sets of binding sites. Two straight lines were obtained for $i = 1$ and $i = 2$ of Eq. 4 with negative slopes, $-K_1$ and $-K_2$, indicating that both postulated classes of binding sites are saturable, one more readily than the other. The values obtained for the respective binding parameters were $K_1 = 1.00 \times 10^6/M$, $n_1P_1 = 1.60 \times 10^{-6}$ M, $K_2 = 8.44 \times 10^3/M$, and $n_2P_2 = 2.25 \times 10^{-5}$ M.

Plasma Protein Binding of Disopyramide and Its Monodealkylated Metabolite in Presence of Each Other—The metabolite data could be fitted by a straight line in the modified Scatchard plot of Fig. 2 and indicated that the metabolite may be bound to one class of binding sites without mutual interaction within the concentration range studied. The binding parameters were $K_M = 5.39 \times 10^3/M$ and $n_MP_M = 2.55 \times 10^{-5}$ M. When disopyramide was added at the relatively high concentration of 2.94×10^{-4} M for a molar ratio of metabolite to parent drug of 0.080, the ability of plasma protein to bind the metabolite was significantly decreased.

The protein binding characteristics of disopyramide in the absence and presence of the metabolite (II) are shown in the modified Scatchard plots of Fig. 2. The molar concentration ratios of parent drug and metabolite were varied between 0.0034 and 7.20. At high concentrations of the metabolite (5.08×10^{-4} M) and low molar ratios of parent drug to metabolite (0.0034, 0.014, 0.14, and 0.84), the binding capacity of the plasma protein for disopyramide was significantly decreased and the data points of the parent drug were fitted by a single straight line where $K_3 = 7.98 \times 10^3/M$ and $n_3P_3 = 2.38 \times 10^{-5}$ M. This indicated that, on the premise of two classes of independent binding sites, the availability of a more readily saturable class of binding sites was decreased. There appeared to be no significant interaction between parent drug and metabolite at lower concentrations of the metabolite (2.54×10^{-6} -5.08×10^{-7} M) where the molar ratios of parent drug to metabolite were higher (7.20, 3.37, and 2.70). These latter circumstances corresponded to those found in pharmacokinetic studies of disopyramide in humans[2].

These facts imply that the parent drug may be bound to two different classes of independent sites, or to at least one class of binding sites where the binding of one molecule of the drug decreases the affinity of another to a previous equivalent binding site. The binding of the metabolite, studied only over a limited concentration range since the available radiolabeled specific activity was low, was consistent with binding to a single class of independent sites. The binding parameters (K_m and n_mP_m) were similar to those obtained for the parent drug (K_2 and n_2P_2) for the less saturable class of binding sites or for all concentrations of disopyramide in the presence of high metabolite concentration (K_3 and n_3P_3).

The interaction between disopyramide and its metabolite that affects the protein binding of each other can be explained by a competition for common binding sites or by conformational changes of the protein molecule induced by the binding of one compound to its specific binding site that modifies or inhibits the binding of the other to its own.

Table II—Effect of Temperature on Plasma Protein Binding of Disopyramide from Equilibrium Dialysis

Disopyramide Concentration	Fraction of Disopyramide Bound	
	37°	25°
2.89×10^{-4}	—	0.07
2.68×10^{-4}	0.00	—
1.42×10^{-4}	0.04	—
1.31×10^{-4}	—	0.09
9.24×10^{-6}	0.36	0.35
2.90×10^{-6}	0.50	0.43

Table III—Effect of Concentration, Time, and Presence of the Monodealkylated Metabolite on Partition Coefficient, D, of Disopyramide into Red Blood Cells as Analyzed after Centrifugation of Red Blood Cell Suspensions in Plasma Water

Time after Spiking, min	10^5 $[A_B]$ Disopyramide	10^4 $[A_B]$ Metabolite	D[a]
5	15.8		1.30
33			1.24
63			1.19
Average			1.24 ± 0.050
5	15.8	4.50	1.21
33			1.19
63			1.30
Average			1.23 ± 0.053
5	8.23		1.25
9			1.23
Average			1.24 ± 0.014
5	8.23	4.74	1.24
9			1.31
Average			1.27 ± 0.050
6	0.823		1.13
18			1.27
33			1.28
Average			1.23 ± 0.080

[a] $D = [A_{RBC}]/[A_{p^u}]$ is the ratio of disopyramide concentration in the erythrocytes to the concentration of free or unbound drug in the plasma.

Influence of pH on Plasma Protein Binding of Disopyramide—The plasma protein binding of disopyramide was the same for plasma pH values of 7.4 and 8.0 but decreased significantly at pH 6.7 for the entire studied concentration range of 2.74×10^{-4}–2.70×10^{-6} M (Table I).

The fact of decreased binding capacity at pH 6.7 may be most readily explained by a net increase in positive charge on the protein to inhibit the binding of the protonated disopyramide. Of course, lowering of the pH to 6.7 could cause conformational changes in the interacting protein fractions (21) that lessen binding affinity.

The fact that there was no systematic decrease of binding with pH indicates that it is difficult to explain the observed data by assuming that only unionized drug (pKa′ = 10.45) is bound, since the ratios of unionized to ionized species would be 0.00017 at pH 6.7, 0.001 at pH 7.45, and 0.0036 at pH 8.0.

Influence of Temperature on Plasma Protein Binding—No significant difference in f, the fraction of disopyramide bound to plasma proteins, at pH 7.45 was found between 25 and 37° using equilibrium dialysis (Table II). Small temperature dependence of binding is characteristic for an interaction between oppositely charged species (22).

Red Blood Cell Partitioning of Disopyramide—The red blood cell–plasma partition coefficient of a drug, on the presumption that only unbound drug in the plasma can diffuse into the red blood cells, can be defined (17) as:

$$D = [A_{RBC}]/[A_{p^u}] = \{[A_B]/[A_{p^u}](1 - H) - [A_{p^b}]/[A_{p^u}] - 1\}(1 - H)/H \quad \text{(Eq. 6)}$$

Table IV—Average Values of the Partition Coefficient, D ($\pm\sigma$), for the Partitioning of Unbound Disopyramide in Plasma into Red Blood Cells[a]

1	2	3	4	5
1.24 ± 0.050	1.17 ± 0.15	1.12 ± 0.14	1.10 ± 0.12	1.04[b] ± 0.06

[a] 1 = Red blood cell–plasma water suspensions by liquid scintillation spectrophotometry, after separation by centrifugation; 2 = whole blood by spectrofluorometry, after separation by centrifugation; 3 = red blood cell–phosphate buffer suspensions by spectrofluorometry, after separation by centrifugation; 4 = whole blood by spectrofluorometry, after separation by ultrafiltration; and 5 = repartitioning from red blood cells into plasma water by liquid scintillation spectrophotometry after separation by centrifugation. [b] Resultant $[A_B] = 3.20 \times 10^{-5}$ M.

$$D = [A_{RBC}]/[A_p{}^u] = \{[A_B]/[A_p]\}(1 - f)(1 - H) - f/(1 - f) - 1\}(1 - H)/H \quad \text{(Eq. 7)}$$

where $[A_{RBC}]$ is the total drug concentration in the red blood cells; $[A_p{}^u]$ and $[A_p]$ are the drug concentrations in plasma, free and total, respectively; $[A_B]$ is the total concentration of a drug in whole blood or red blood cell suspension; H is the hematocrit; $(1 - H)$ is the plasmacrit; and f is the fraction of drug bound to plasma protein.

In a red blood cell–buffer or red blood cell–plasma water suspension, D can be calculated from Eq. 6 where $[A_p{}^b] = 0$. The consistency of the partition coefficients of disopyramide (Table III) with time after spiking of red blood cell suspensions in plasma water shows that the equilibrium of drug between plasma water and red blood cells is reached in less time than is needed for the performance of the initial mixing and subsequent centrifugation of the samples. The presence of high concentrations of the metabolite to give molar ratios of 0.35 and 0.17 of disopyramide to the metabolite had no significant effect on disopyramide partitioning. The values for $D = [A_{RBC}]/[A_p{}^u]$ were constant and concentration independent. However, if a partition coefficient were obtained for the partitioning of disopyramide between total plasma concentration and red blood cell concentration so that $D^* = [A_{RBC}]/[A_p]$, the resultant values would be concentration dependent and would decrease as f increases. For example, D^* at $f = 0.10$ is 1.11 but at $f = 0.33$ and 0.65, it would be 0.82 and 0.45, respectively.

When the values of D were obtained from disopyramide addition to whole blood and the fraction bound, f, was taken into account (Eq. 6), excellent agreement was shown (Table IV) with the values obtained from plasma–red blood cell and phosphate buffer–red blood cell suspensions. It was shown also that when disopyramide was repartitioned from red blood cells into plasma water, the obtained D values were the same.

The partition coefficients, D, for disopyramide were consistently larger than 1.0. This demonstrates an additional binding to, or greater solubility in, the red blood cells than can be accounted for on the assumption that the volume of the erythrocytes contains only an aqueous phase wherein the disopyramide has the same chemical activity as in plasma water.

Table V—Effect of Concentration, Time, and Presence of Disopyramide on the Partition Coefficient, D, of the Monodealkylated Metabolite into Red Blood Cells as Analyzed after Centrifugation of Red Blood Cell Suspension in Plasma Water

Time after Spiking, min	$[A_B]$ Metabolite	$[A_B]$ Disopyramide	D^a Metabolite
8	1.20×10^{-4}		0.81
36	1.20×10^{-4}		1.34
66	1.20×10^{-4}		1.42
8	1.20×10^{-4}	2.56×10^{-4}	0.82
36	1.20×10^{-4}	2.56×10^{-4}	1.37
66	1.20×10^{-4}	2.56×10^{-4}	1.43
5	8.00×10^{-5}		0.96
9	8.00×10^{-5}		1.30
66	8.00×10^{-5}		1.42
4.5	4.00×10^{-5}		0.13
7	4.00×10^{-5}		1.19
14	4.00×10^{-5}		1.12

[a] $D = [A_{RBC}]/[A_p{}^u]$ is the ratio of the monodealkylated metabolite concentration in the erythrocytes to the concentration of free or unbound drug in plasma. The average value of D at 66 min was 1.42 ± 0.005. On repartitioning of red blood cells containing monodealkylated disopyramide, the obtained D was 1.23 after 66 min, when the resultant $[A_B] = 3.30 \times 10^{-5}$ M.

Red Blood Cell Partitioning of Metabolite—Equilibration of the monodealkylated metabolite between red blood cells and plasma was reached after a significant delay (Table V) and appeared to be independent of metabolite concentration. The presence of disopyramide for a molar ratio of 0.47 of metabolite to parent drug did not affect the partitioning of the metabolite. An equivalent result was obtained when the metabolite was partitioned from the red blood cells into the plasma. The average D value of 1.4 obtained for the metabolite was indicative of an additional binding and/or sequestration over simple distribution into the water phase of the red blood cells.

The difference between equilibrium between plasma and red blood cells for disopyramide and metabolite may be explained by the fact that the metabolite has reduced lipophilicity due to its lessened number of alkyls, which may significantly slow down its diffusion through the lipid-like membrane of the red cells (23).

Pharmacokinetic Implications—Since the protein binding of disopyramide is concentration dependent, the pharmacokinetics of disopyramide, when assayed as total concentration in plasma, could not be linear if only the free fraction of drug in plasma diffuses, is excreted, or is metabolized by first-order processes. However, such kinetics are more amenable to linearization if the pharmacokinetic model is based on concentration of unbound drug in the plasma and if the overall elimination processes are assumed to be first order. This hypothesis was challenged and confirmed by the fact that red blood cell partitioning coefficients were invariant only when based on the premise that the unbound fraction of drug in the plasma partitions. In essence, this corresponds to an isolated two-compartment system *in vitro*, which would be difficult to evaluate *in vivo*.

The determination of a time-dependent partitioning of a drug into red blood cells is vital for establishing a valid pharmacokinetic model. It is apparent that the blood compartment cannot be considered as kinetically homogeneous with respect to the metabolite. The red blood cells can actually represent a kinetically definable separate compartment with slow drug influx, a fact that must be considered in the forthcoming pharmacokinetic analysis.

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Urinary Excretion of Amitriptyline *N*-Oxide in Humans

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Abstract □ The excretion rate of amitriptyline *N*-oxide was measured in healthy human subjects after the administration of a single oral dose of amitriptyline. The total amount of this metabolite excreted accounted for about 1% of the dose. It appears in the urine soon after administration of the parent compound but has practically disappeared by the 9th hr after treatment. The role of this metabolite in amitriptyline degradation in humans is discussed.

Keyphrases □ Amitriptyline—excretion of *N*-oxide urinary metabolite in humans □ Metabolism—urinary excretion of amitriptyline *N*-oxide in humans □ Biotransformation—urinary excretion of amitriptyline *N*-oxide in humans

Excretion of the major metabolites of amitriptyline and related drugs has been extensively studied in humans (1, 2). There exists, however, an interesting minor metabolic route, that of *N*-oxidation. The mechanism of formation, metabolism, and excretion of these *N*-oxides was investigated (3–6) both *in vitro* and *in vivo* in rats and guinea pigs. However, in humans, studies of the excretion of these compounds have, thus far, been limited to imipramine *N*-oxide (7, 8).

Because of previous work in this laboratory on amitriptyline metabolism, interest also existed in its metabolism to the *N*-oxide (9). According to Bickel and coworkers (3–6), some reasons why this minor pathway is of general interest are:

1. The natural occurrence of *N*-oxides in plant and animal tissues has posed interesting problems as to the biochemistry and function of these compounds in biological systems.

2. *N*-Oxides are metabolites of many tertiary amine drugs and have been postulated as intermediates in *N*-dealkylation.

3. Certain *N*-oxides possibly formed in mammalian tissues act as antimetabolites or carcinogens and are postulated as possible inducers of spontaneous cancer (10).

This paper reports studies on the excretion rate of amitriptyline *N*-oxide in humans. On the basis of these data and the results of previous work (9) on amitriptyline metabolism in humans, the probable role of this metabolite in the degradative pathway of the drug is discussed.

EXPERIMENTAL[1]

Synthesis of Amitriptyline *N*-Oxide—The synthesis of amitriptyline *N*-oxide was performed according to the method of van Steen (11), with the following modifications. Five grams of amitriptyline base, 10,11-dihydro-*N*,*N*-dimethyl-5*H*-dibenzo[*a*,*d*]cycloheptene-$\Delta^{5\gamma}$-propylamine, was dissolved in 20 ml of anhydrous methanol. Then 5 ml of 30% H_2O_2 was added to the solution, dropwise with stirring, and the solution was kept at 0° for a few days. After the further addition of 3 or 4 ml of 30% H_2O_2 and subsequent storage at 0° for 5 days, the excess hydrogen peroxide was cautiously decomposed with an aqueous suspension of 10% Pt black. This mixture, kept in ice, was freed from Pt black by filtration, and the filtrate was concentrated under reduced pressure at 30°. The solid residue was twice recrystallized from a mixture of methyl chloride–anhydrous ether (1:1) and colorless crystals of pure amitriptyline *N*-oxide, mp 114–115°, were obtained in an 88% yield. Amitriptyline *N*-oxide in the original method of van Steen melts at 99–106°. The IR spectrum of amitriptyline *N*-oxide was identical to that of amitriptyline except for the absence of the signal of the NH-band between 2400 and 2550 cm^{-1}. The UV spectrum of the compound, dissolved in methanol, showed the absorption maximum at 238 nm characteristic of amitriptyline.

Characterization of Amitriptyline *N*-Oxide—*TLC*—Glass plates (10 × 20 cm) were spread with a mixture of silica gel G[2]–fluorescent indicator[3]–distilled water (1:0.04:2); the thickness was 0.3 mm. Standards of amitriptyline *N*-oxide, amitriptyline, and the other amitriptyline metabolites known to occur in humans (1) (10-hydroxyamitriptyline, nortriptyline, desmethylnortriptyline, and 10-hydroxynortriptyline) were chromatographed by the ascending technique in the following solvent systems: A, isopropanol–ethyl acetate–concentrated ammonium hydroxide (45:5:1); B, chloroform–methanol (4:1); and C, benzene–dioxane–water–concentrated ammonium hydroxide (62.5:35:1.8:0.7).

The detection of amitriptyline and its metabolites was performed with a UV light (254 nm); the R_f values are listed in Table I. In all solvent systems tested, amitriptyline *N*-oxide possessed

[1] All chemicals and reagents (analytical grade) were obtained from Merck, Darmstadt, Germany. Amitriptyline, nortriptyline, desmethylnortriptyline, 10-hydroxyamitriptyline, and 10-hydroxynortriptyline were supplied by Merck, Sharp & Dohme, Pavia, Italy.
[2] Merck, Darmstadt, Germany.
[3] Woelm, Eschwege, Germany.