Protein Binding and Erythrocyte Partitioning of the Antirheumatic Proquazone

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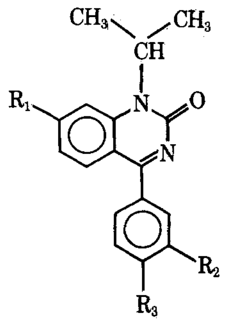
Abstract □ The kinetics of proquazone, a new nonacidic nonsteroidal anti-inflammatory drug, were investigated by equilibrium dialysis and red blood cell partitioning methods on human blood and its subcompartments: erythrocytes, plasma, and plasma water. The binding of this lipophilic compound to plasma proteins and albumin was high (98%) and was not concentration dependent or altered in the presence of large concentrations of metabolites. The plasma protein binding of proquazone increased with increasing pH. The apparent solubility of the hydrophobic drug was largely increased in buffers in which albumin was admixed in high concentrations. Albumin as a biological solubilizer permits intravenous administration of significantly larger amounts of the drug. The erythrocyte–buffer partition coefficient averaged 5.5 and was pH dependent. Equilibrium between red blood cells and the buffer was obtained quickly after drug addition (<2 min). The erythrocyte–plasma partition coefficient value of 0.09 indicated that only unbound drug partitions into red cells.

Keyphrases □ Proquazone—erythrocyte partitioning and equilibrium dialysis methods evaluated for determination of plasma protein binding of drug □ Protein binding—proquazone in human plasma, erythrocyte partitioning and equilibrium dialysis methods evaluated □ Anti-inflammatory agents—proquazone, erythrocyte partitioning and equilibrium dialysis methods evaluated for determination of proquazone binding to plasma proteins

Proquazone[1], a new anti-inflammatory compound (1, 2), exhibits antiphlogistic activities in animals and humans comparable to those of classical, nonsteroidal anti-inflammatory compounds (1–4). Its primary therapeutic indications are thought to be in the treatment of rheumatoid arthritis, osteoarthritis, and gouty arthritis (5, 6). However, proquazone differs chemically from other representatives of this drug class. It is a quinazolinone derivative (I) and a weak base[2]. Proquazone is strongly lipophilic[3] with low aqueous solubility[4].

The performance and analysis of the pharmacokinetics of highly lipophilic compounds may cause special problems. Intravenous experiments have not been performed with proquazone. Its low water solubility necessitates either administration of smaller dosages in aqueous solution or higher dosages dissolved in large and, thus, toxic quantities of solubilizers (*e.g.*, glycerolformal[5]). Preliminary pharmacokinetic experiments with proquazone administered orally to healthy subjects showed that this drug is metabolized extensively[5]. The metahydroxy- (II), methylhydroxymetahydroxy (III), methylhydroxy- (IV), and carboxylic acid (V) metabolites were identified as major metabolites in humans[5].

The primary aim of this study was to determine proquazone kinetics *in vitro* in the blood subcompartments: plasma, plasma water, and erythrocytes. The results are



I: $R_1 = CH_3, R_2 = R_3 = H$
II: $R_1 = CH_3, R_2 = OH, R_3 = H$
III: $R_1 = CH_2OH, R_2 = OH, R_3 = H$
IV: $R_1 = CH_2OH, R_2 = R_3 = H$
V: $R_1 = COOH, R_2 = R_3 = H$

prerequisites for a proper delineation of the pharmacokinetics of the drug *in vivo*. Another aim was to develop an appropriate nontoxic intravenous dosage form for the administration of sufficiently large proquazone dosages so that its intravenous kinetics could be determined using the fluorometric assay developed[6].

EXPERIMENTAL

Reagents—Proquazone[1] (I), [^{14}C]proquazone[1] (^{14}C-I), and metahydroxy[1] (II), 7-methylhydroxymetahydroxy[1] (III), 7-methylhydroxy[1] (IV), and 7-carboxylic acid[1] (V) metabolites were used. The specifically labeled ^{14}C-I ([4-*phenyl*-3,5-^{3}H]proquazone) had a specific activity of 79.0 μCi/mg. Its radiochemical purity exceeded 96% in three different TLC systems: chloroform–methanol (95:5) developed on silica gel[7], ethyl acetate developed on silica gel[7], and ether developed on silica gel[7].

Heparinized (5 USP units/ml) blood and human plasma with known protein fractions were obtained from healthy volunteers (Subjects A–I), who had no prior drug intake. Human albumin[8,9] and hemoglobin[10] were purchased. A human albumin solution used for blood volume expansion in the treatment of shock was obtained from a hospital pharmacy[11]. Different 0.067 *M* phosphate buffers (8) were prepared to which sodium chloride was added to give an osmolarity of 0.300 Osmol (pH 6.60–8.00). The ionic strength of the buffers varied between 0.19 and 0.27. The phosphate buffers were used for preparation of the erythrocyte buffer suspensions and the albumin and hemoglobin solutions.

Instruments—Hematocrits of erythrocyte suspensions were determined with a centrifuge[12]. A two-chamber dialysis apparatus with chamber volumes of 1.2 ml each, separated by a membrane[13], was used for equilibrium dialysis of plasma and the albumin and hemoglobin solutions. Ultrafiltration of the albumin solutions was effected with high-flux cone membranes[14].

Liquid Scintillation Counting—Plasma, plasma water, and buffer

[1] Biarison, Sandoz Ltd. and Wander Ltd., Switzerland.
[2] An apparent pKa of 1.1 was obtained for proquazone in methyl ether cellulose in water (70:30 v/v) by the titrimetric method of Albert and Serjeant (7).
[3] An octanol–water partition coefficient of 13.2 was obtained for proquazone at a pH of 1.2 for the aqueous phase and room temperature.
[4] Proquazone solubility in water is ≤0.1% (g/v).
[5] Unpublished data.

[6] Lower level of sensitivity in plasma and urine is 10 ng/ml; to be published.
[7] Silica gel 60 F_{254}, 250 μm, Merck, Darmstadt, West Germany.
[8] Fraction V, fatty acid free, No. A-1887, Miles Laboratories, Elkhart, Ind.
[9] Fraction V, fatty acid free, No. A-1887, Sigma Chemical Co., St. Louis, Mo.
[10] Type IV, crystallized twice, No. H-7379, Sigma Chemical Co., St. Louis, Mo.
[11] Twenty percent (g/v) human albumin SRK, Berne, Switzerland.
[12] Readacrit, Clay Adams, Division of Becton–Dickinson, Parsippany, N.J.
[13] Cuprophane, Technicon Ltd., Zürich, Switzerland.
[14] Centriflo 50 membrane ultrafilters with conical supports and tubes, Amicon Corp., Lexington, Mass.

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were assayed for radioactivity directly or after combustion. The radioactivity in erythrocytes was measured by liquid scintillation counting[15] only after hemolysis and combustion. Hemolysis was induced by keeping the samples frozen at −20° for 2 hr. Combustion was effected with a sample oxidizer[16]. The efficiency of combustion was monitored by simultaneous processing of biological samples spiked with known amounts of radioactivity.

Aliquots (100–500 μl) of plasma, plasma water, and buffer were transferred to liquid scintillation vials to which 3 ml of liquid scintillation fluid[17] was then added. After the contents were mixed thoroughly, the vials were kept at 37° for 2 hr to allow the water to dissolve in the liquid scintillation fluid. Replicate samples of 100–500 μl of buffer, plasma, and hemolyzed erythrocytes were oxidized to tritiated water, which was subsequently dissolved in Bray's liquid scintillation fluid[18] (9). The vials were kept for 2 hr at 4° in the dark prior to counting. The measured radioactivity was corrected for background and loss of efficiency by relation to an external standard. Separate quench curves were constructed for the two liquid scintillation fluids.

Protein Binding of I—Equilibrium dialysis, erythrocyte partitioning, and ultrafiltration were used for the determination of ^{14}C-I binding to proteins. The techniques used in equilibrium dialysis were described previously (10). Dialysis of the protein solutions spiked with ^{14}C-I was performed against buffer for 4 hr. Preliminary experiments showed that complete equilibration of free drug in both chambers of the apparatus occurred during this time. All experiments were performed at 37° at pH 7.35–7.45 unless otherwise specified.

The protein binding of ^{14}C-I was investigated in healthy subjects (Subjects A–I). Subjects A–F also participated in the pharmacokinetic study[19]. The albumin and globulin contents of all plasma were determined (11, 12) and were within the physiological ranges of 42–54 and 23–31 g/liter, respectively (13). The plasma protein binding of ^{14}C-I was studied over the 50–50,000-ng/ml range found for the drug in pharmacokinetic studies after intravenous dosages of 75 and 122 mg and oral dosages of 300 and 900 mg. The plasma protein binding of ^{14}C-I in the presence of Metabolites II–V was investigated at a total metabolite to drug ratio of 20:1.

Since the pH in inflamed tissue reportedly varies (14, 15), the plasma protein binding of ^{14}C-I was investigated in the pH range of 6.60–8.30. These experiments were performed in the presence and absence of II–V. The influence of temperature on plasma protein binding of ^{14}C-I was investigated at 37 and 22°. The albumin binding of ^{14}C-I was studied at various albumin concentrations (0.5–50 mg/ml, 7.25–725 × 10^{-6} mole/liter) and ^{14}C-I concentrations (60–60,000 ng/ml, 2.16–3370 × 10^{-8} mole/liter). The purpose of these experiments was to determine the binding parameters of ^{14}C-I at an albumin concentration where the binding of ^{14}C-I is clearly saturable and to investigate the binding of ^{14}C-I at albumin concentrations comparable to those in the interstitial and synovial fluids (16–18). All of these experiments were performed at 37°, and the pH was maintained at 7.35–7.45 unless otherwise specified. Constancy of the pH was ascertained by measurement before and after dialysis.

Solubility Analysis and Protein Binding of I—Preliminary experiments established that the concentrated albumin (dissolved in buffer) could be employed as a "biological solubilizer" for the hydrophobic drug, I. These experiments indicated that the drug was highly bound to albumin at "physiological" albumin concentrations (40–50 mg/ml) and that the bound amounts of I were augmented significantly when the volumes or concentrations of the albumin solution were increased. Quick estimates of the maximum amounts of I soluble in albumin solutions at 22° were obtained by determining the concentrations of I in increasingly concentrated albumin solutions that just provoked microscopically visible drug precipitation. These I concentrations ranged between 4.0 and 5.0 mg/ml of albumin solution (200 mg/ml). This protein concentration was the highest that could be safely given intravenously.

For more definitive determinations of quasimaximum dosages for intravenous application, a centrifugal method and modified ultrafiltration were employed. With the centrifugation method, test tubes were filled with 5 ml of albumin solution (200 mg/ml) spiked with ^{14}C-I dissolved in glycerolformal (2–4% v/v) to give final ^{14}C-I concentrations of 1.6, 2.4, and 3.2 mg/ml. After mixing for several hours at 22°, the solutions were centrifuged at 6000×g for 10 min. Five consecutive aliquots (1 ml) then were taken, starting from the top and proceeding to the bottom of the tubes. The radioactivity in each aliquot was measured separately. Supersaturation of the albumin solution and precipitations of ^{14}C-I would lead to significantly increased activities in the aliquots from the bottom of the tubes.

In the modified ultrafiltration procedure, unbound concentrations of ^{14}C-I were determined in albumin solutions (174 mg/ml) spiked with ^{14}C-I in glycerolformal (4% v/v) to yield total drug concentrations of 3.0 mg/ml. A solubility "reserve" could be estimated from the difference between the known maximal concentration of ^{14}C-I soluble in aqueous solution and the unbound concentration of ^{14}C-I found with the albumin solution. Ultrafiltration was preferred over equilibrium dialysis because of potential bias in drug binding estimates at high protein concentrations with the latter method. Net transport of water from the buffer to the protein-containing chambers as a result of an osmotic pressure difference may lead to a considerable decrease in the effective protein concentration during equilibrium dialysis. Ultrafiltration experiments were performed at 22° with a pH 6.8 human albumin solution[9].

The original ultrafiltration procedure (19) was modified slightly. Presaturation of the cone membranes with ^{14}C-I was necessary after preliminary experiments demonstrated significant concentration-dependent and saturable membrane binding of ^{14}C-I. All cone membranes used in the final experiments thus were presaturated routinely with I by filtration of the albumin solution to one-tenth of the volume after spiking with the appropriate concentration of ^{14}C-I. Successful saturation of the membranes was demonstrated when cones that were repetitively (one to six times) filled with fresh albumin solution spiked with 3.0 mg of ^{14}C-I/ml showed constant recoveries in the second to sixth filtrate after 10 filtrations. The 10th filtrate of the albumin solutions was reached after centrifugation for 10 min at 2500×g. Complementation of the ultrafiltration procedure by equilibrium dialysis proved to be necessary after the filtrates were shown to contain albumin in concentrations that bound ^{14}C-I significantly. The true unbound concentrations of ^{14}C-I in the filtrates could be determined in the buffer chambers after they were subjected to equilibrium dialysis.

Erythrocyte Partitioning of I—The determination of the erythrocyte partitioning of ^{14}C-I was performed in erythrocyte–buffers and erythrocyte–plasma suspensions having constant hematocrits of 40%. Erythrocytes for suspension in buffer were obtained by centrifugation of fresh whole blood. After removal of the upper layer containing the buffy coat, the red blood cells were washed twice with twice the volume of 0.9% NaCl solution and once with twice the volume of buffer. After further centrifugation, aliquots of erythrocytes were added to the buffer to yield hematocrits of 40%. Erythrocyte–plasma suspensions were obtained similarly. The buffy coat was removed from plasma after centrifugation of blood. The erythrocytes obtained were resuspended in plasma without prior washing.

The red cell–buffer partitioning of ^{14}C-I was studied over a concentration range of 50–5000 ng/ml in the presence and absence of unlabeled Metabolites II–V. The ratio of total metabolite to drug was 20:1. All experiments were performed at 37° at pH 7.30–7.45 unless otherwise

Table I—Percentage of Plasma Unbound Proquazone, φ, in the Presence and Absence of Its Metahydroxy, Methylhydroxymetahydroxy, Methylhydroxy, and Carboxylic Acid Metabolites in Plasma from Subject F

Total Plasma Proquazone Concentration (C_p), ng/ml	Dialysis	φ	Mean ± *SD* (n = 3)
50	1	1.65	
	2	1.69	1.68 ± 0.023
	3	1.69	
300	1	1.78	
	2	1.79	1.78 ± 0.058
	3	1.78	
300[a]	1	1.85	
	2	1.82	1.84 ± 0.015
	3	1.84	
2500	1	1.86	
	2	1.80	1.81 ± 0.050
	3	1.76	
5000	1	1.74	
	2	1.75	1.74 ± 0.058
	3	1.74	
Overall mean ± *SD* (n = 15)			1.77 ± 0.061

[a] The concentration ratio, 20, is the sum of unlabeled metabolites to labeled proquazone.

[15] Packard Tri-Carb 3280 and 3255, Packard Instruments, Downers Grove, Ill.
[16] Model 4101, Intertechnique, Plaisir, France.
[17] Instagel, Packard-Becker, BV, Groningen, The Netherlands.
[18] Bray's solution: 700 ml of 1,4-dioxane, 300 ml of toluene, 20 g of naphthalene, and 7 g of butyl 2-phenyl-5-(4-biphenyl)-1,3,4-oxadiazole.
[19] P. H. Hinderling and A. Roos, to be published.

Table II—Percentage of Plasma Unbound Proquazone, φ, in the Presence and Absence of Its Metahydroxy, Methylhydroxymetahydroxy, Methylhydroxy, and Carboxylic Acid Metabolites in Plasma of Subjects A–F

Total Plasma Proquazone Concentration (C_p), ng/ml	Percentage of Plasma Unbound Proquazone, φ					
	Subject A	Subject B	Subject C	Subject D	Subject E	Subject F
300	1.96 ± 0.070	1.82 ± 0.046	1.95 ± 0.015	2.09 ± 0.082	2.04 ± 0.053	1.78 ± 0.006
300[a]	2.04 ± 0.036	—	—	—	—	1.84 ± 0.015
5000	2.06 ± 0.040	1.99 ± 0.026	2.12 ± 0.067	2.29 ± 0.010	2.05 ± 0.055	1.74 ± 0.006
Overall mean	2.02	1.90	2.04	2.19	2.05	1.79
SD	± 0.063	± 0.101	± 0.099	± 0.120	± 0.049	± 0.044
n	9	6	6	6	6	9

[a] The concentration ratio, 20, is the sum of unlabeled metabolites to labeled proquazone.

specified. The constancy of pH was ascertained by the equivalency of the values obtained at the start and finish of the experiments. The erythrocyte–buffer partition coefficient of ^{14}C-I in the suspensions was determined at 10, 30, 60, and 90 min after addition of I. In another experiment, the partitioning of ^{14}C-I was studied systematically as a function of time over 140 min; the apparent partition coefficient was determined at 2, 4, 6, 9, 14, 20, 90, 120, and 140 min following addition of I.

In other experiments, the erythrocyte partitioning of ^{14}C-I was studied as a function of pH. Red blood cell–buffer suspensions were prepared at pH values of 6.60, 6.90, 7.20, and 7.50, and the partitioning of ^{14}C-I then was determined 30 and 60 min after I addition. The concentrations of ^{14}C-I were determined in both phases of the suspensions, in the apparent red blood cell phase and in the true buffer or plasma phase. Aliquots were taken and centrifuged at 2500×*g* at intervals of 0.5–5 min, and the true buffer or plasma phases were removed. Hematocrits were determined in the remaining apparent erythrocyte phases; they ranged between 60 and 95%.

The binding of ^{14}C-I to hemoglobin was investigated since slight hemolysis occurred in the erythrocyte–buffer partitioning experiments. The hemoglobin concentrations then were measured routinely[20] and ranged between 0 and 25 mg/ml (0–3.62 × 10^{-5} mole/liter). If no corrections were applied for the hemoglobin-bound ^{14}C-I in the buffer phase, then the partition coefficients for the drug would be underestimated.

Erythrocyte partitioning was also employed as an alternative method for measuring the plasma protein binding of ^{14}C-I. For comparison, the protein binding of ^{14}C-I in plasma of Subject I was assayed simultaneously by partitioning and equilibrium dialysis.

RESULTS AND DISCUSSION

Protein Binding of Proquazone[21] (I)—The percentage of drug unbound to plasma protein, albumin, or hemoglobin, φ, was calculated according to:

$$\varphi = 10^2 - \beta = 10^2\left(1 - \frac{C_b}{C_t}\right) = 10^2\left(\frac{C_u}{C_t}\right) \quad \text{(Eq. 1)}$$

The binding of ^{14}C-I to plasma protein and albumin at "physiological" concentrations of 70 and 50 mg/ml of albumin was relatively large. The ^{14}C-I concentration-independent φ value amounted to only 2% on the average (Tables I and II). The φ value was unaltered by the presence of large concentrations of Metabolites II–V (Tables I and II). There was a remarkably small variability of φ for a given individual plasma. The maximum intersubject difference in φ for the subjects tested was 18% (Table II). Equivalent φ values were obtained for ^{14}C-I (50–50,000 ng/ml) with plasma and albumin (50 mg/ml). On the average, φ was 1.99 ± 0.155 (n = 36) and 1.98 ± 0.113 (n = 6), respectively. Large concentrations of metabolites did not influence the binding of ^{14}C-I to albumin [φ = 1.97 ± 0.120 (n = 2)].

These results indicated that ^{14}C-I was bound predominantly to the albumin fraction in plasma. The extent of plasma protein binding was clearly pH dependent (Fig. 1). There was an apparent linear relationship between φ and pH. The φ values at pH 7.80 averaged 1.40 but were 2.30 at pH 7.00. This difference was equivalent to a 40% rise in concentration of the pharmacologically active unbound drug species when the pH was lowered by 0.80 unit. The pH-dependent binding characteristics of ^{14}C-I were not influenced by the presence of large concentrations of II–V (Fig. 1). These findings may be clinically relevant if proquazone binding to synovial and interstitial proteins are similarly pH dependent. It has been reported that the pH is low and the protein content is increased in chronically inflamed synovial tissues and fluids (15, 16).

The plasma protein binding of ^{14}C-I (5000 ng/ml) was temperature independent between 22 and 37°: φ (37°) = 2.04 ± 0.171 (n = 18) and φ (22°) = 2.05 ± 0.071 (n = 2). A gradual decrease of the albumin concentrations from 50 to 0.5 mg/ml (725–7.25 × 10^{-6} mole/liter) with a constant concentration of ^{14}C-I (300 or 50,000 ng/ml, 1.08 or 18.0 × 10^{-7} mole/liter) brought a gradual increase of φ (Table III). The φ value was identical for both ^{14}C-I concentrations at a given albumin concentration in the range between 10.0 and 50.0 mg/ml (14.5–72.5 × 10^{-5} mole/liter) (Table III). Scatchard plots with an apparent positive slope (Fig. 2) characterized the binding behavior of I when the protein concentration was varied within the range of 7.25–72.5 × 10^{-5} mole/liter. At the lowest albumin concentration of 0.5 mg/ml (7.25 × 10^{-6} mole/liter), φ clearly varied with the ^{14}C-I concentrations, and the binding was saturable (Table III and Fig. 3).

Proquazone is a lipophilic compound. Extensive binding to plasma protein and albumin has been reported for other lipophilic drugs (21, 22). Proquazone is a weak base with an apparent pKa of 1.1. Its unionized species largely prevails at the pH range studied. The compound posesses two partial charges in the quinazolinone ring: a negative charge at the oxygen of the carbonyl group and a positive charge at the adjacent nitrogen. The decrease of φ with increasing pH suggests that hydrophobic rather than electrostatic forces or hydrogen bonds are involved in the binding of ^{14}C-I, even though the decrease of φ at higher pH values exceeds the increase of the unionized drug species. Alternatively, a pH- or ionic strength-induced conformational change of plasma protein (albumin) resulting in a larger affinity for the drug may be involved. There is evidence that albumin is a flexible molecule which can undergo reversible conformational alterations (23, 24). Another rationalization may be that

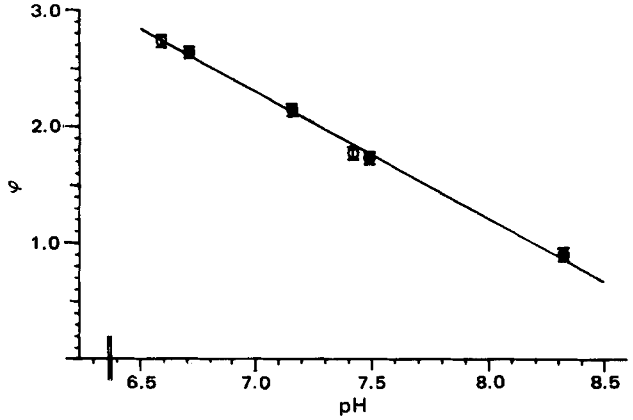

Figure 1—*Apparent pH dependency of percentage of plasma unbound proquazone, φ, in the presence (○) and absence of its metahydroxy, methylhydroxymetahydroxy, methylhydroxy, and carboxylic acid metabolites (●). Values of mean (±SD) (vertical bars) of two experiments performed at each pH level are given. Blood of Subject I was used.*

[20] Hemoglobin after transformation to hemiglobin cyanid was determined spectrophotometrically (20). Calibration curves were set up with Dade HiCN-Standards of Merz & Dade AG, Berne, Switzerland.

[21] The values for φ, unless otherwise specified, were normalized for pH 7.40 according to the experimentally obtained relationship between φ and pH (Fig. 1).

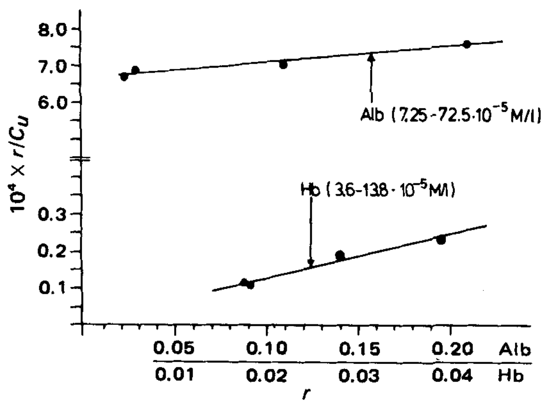

Figure 2—*Classical Scatchard plots of* r/C_u *against* r *with apparent positive slopes for proquazone binding to albumin and hemoglobin. Total concentration of proquazone was 5000 ng/ml (1.80 × 10^{-5} mole/liter). Coefficients and standard errors of linear regressions were for the binding of* ^{14}C-I *to albumin:* r/C_u = *6.67 × 10^4 (±3.81 × 10^2) + 4.48 × 10^4 (±3.15 × 10^3)*r *(coefficient of determination = 0.999); the slope was statistically significantly different from zero [two-sided* t *test:* t *(α = 0.005) = 14.09,* t_{calc} *= 14.21]. Coefficients and standard errors of linear regressions were for the binding of* ^{14}C-I *to hemoglobin:* r/C_u = *1.05 × 10^2 (±4.35) + 5.81 × 10^4 (±1.59 × 10^3)*r *(coefficient of determination = 0.999); the slope was statistically significantly different from zero [two-sided* t *test:* t *(α = 0.001) = 31.60,* t_{calc} *= 36.63].*

a pH- or ionic strength-catalyzed molecular segregation of the protein occurs with an increase of binding surface (25). The ^{14}C-I concentration-independent plasma protein (albumin) binding indicates that there is an abundance of accessible binding sites, which could also explain the observed constancy of φ at high concentration ratios of metabolites to parent drug.

The lowering of albumin concentrations from 50 to 5 mg/ml increased φ but to a lesser extent than was expected. The affinity of albumin for ^{14}C-I at lower protein concentrations (40–10 mg/ml) was clearly larger than at physiological concentrations (50 mg/ml) (Fig. 2). A further decrease in the albumin concentration (0.5 mg/ml) brought a reduction in the affinity for ^{14}C-I. The binding of ^{14}C-I became concentration dependent and saturable (Fig. 3). These findings can be explained by reversible conformational changes (23, 24) or molecular segregation (24) of albumin induced either by dilution or by an increased concentration ratio of the phosphate buffer to the protein. Phosphate buffer was shown to interfere with the binding of small molecules (26). Binding data should not be viewed as a result of the interaction of a ligand and a protein molecule only.

The possible influence of the buffer, its ionic strength and composition, must be evaluated critically. However, there is little choice of buffers that have electrolytic compositions similar to biological fluids and can be used

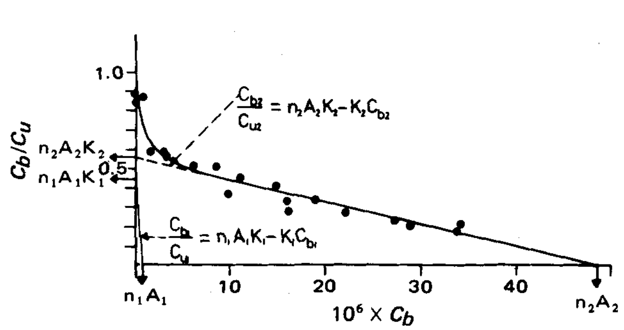

Figure 3—*Modified Scatchard plot for proquazone binding to albumin (0.5 mg/ml = 7.25 × 10^{-6} mole/liter). Total concentrations of proquazone ranged between 50 and 60,000 ng/ml (= 2.16 × 10^{-8}–3.37 × 10^{-5} mole/liter). Graphical analysis according to Rosenthal (35) on the premise of the existence of two independent classes of binding sites was performed. Two straight lines,* $C_{b_1}/C_u = n_1A_1K_1 - K_1C_{b_1}$ *and* $C_{b_2}/C_u = n_2A_2K_2 - K_2C_{b_2}$*, with the slopes of* $-K_1$ *and* $-K_2$*, the respective intercepts on the abscissa of* n_1A_1 *and* n_2A_2*, and the respective intercepts on the ordinate of* $n_1A_1K_1$ *and* $n_2A_2K_2$ *were obtained. The determined apparent binding parameters for proquazone were* $K_1 = 2.0 \times 10^6$ *liters/mole,* $K_2 = 1.25 \times 10^4$ *liters/mole,* $n_1 = 0.027$*, and* $n_2 = 6.35$.

Table III—Albumin Concentration Dependency of the Percentage of Unbound Proquazone, φ

Total Proquazone Concentration (C_p), ng/ml	Albumin, mg/ml	φ, mean ± *SD* (n = 2)
300	50	2.08 ± 0.099
5000		2.01 ± 0.064
300	40	2.44 ± 0.028
5000		2.46 ± 0.035
300	10	8.81 ± 0.120
5000		8.87 ± 0.028
5000	5	15.33 ± 0.410
300	0.5	60.60 ± 1.56
5000		65.69 ± 1.44

in the biologically significant pH range (27). A buffer such as tromethamine interferes with the binding of small molecules (28). The potential impact of contaminants on binding data also should be considered (29). The apparent affinity changes of albumin observed at lowered concentrations are not likely to be due to contaminants or fatty acids found in commercial albumin preparations (29). If this were the case, a decrease in the concentration ratio of contaminant to drug by either lowering the albumin content or increasing the drug concentration should have an equivalent effect on the apparent affinity of the albumin for ^{14}C-I. However, changes in affinity were observed only after dilution of albumin; an increase of the apparent affinity was seen at concentrations between 50 and 5 mg/ml; a decrease of the apparent affinity occurred at 0.5 mg/ml.

Similar apparent affinity increases for ^{14}C-I were observed with differently diluted hemoglobin solutions (9.5–2.5 mg/ml, 13.8–3.60 × 10^{-5} mole/liter) that were spiked with ^{14}C-I to give total concentrations of 300 or 5000 ng/ml. The binding data of ^{14}C-I were characterized by Scatchard plots with positive slopes (Fig. 2). The same rationalizations may be inferred to explain the results presented for the albumin data.

Protein Binding and Solubility Analysis of I—The results of the centrifugation method indicated that ^{14}C-I at total concentrations of 1.6, 2.4, and 3.2 μg/ml was completely dissolved in albumin (200 mg/ml). The radioactivity present in aliquots obtained from the bottom of the tubes was not different from that of the three upper aliquots and was (expressed in percent of the mean activities of the upper three aliquots) 104.1, 99.8, and 101.7% for 1.6, 2.4, and 3.2 μg/ml, respectively, of ^{14}C-I.

The percentage of true unbound ^{14}C-I (φ_{true}) in ultrafiltration and equilibrium dialysis with total concentrations of 3.0 mg of ^{14}C-I/ml of albumin (200 mg/ml) was calculated according to:

$$\varphi_{true} = \frac{\varphi^{UF'}\varphi^{ED}}{10^2} \qquad \text{(Eq. 2)}$$

where $\varphi^{UF'}$ is the apparent percentage of unbound ^{14}C-I in the ultrafiltration procedure and φ^{ED} is the true percentage of unbound ^{14}C-I in the equilibrium dialysis method. The $\varphi^{UF'}$ value was obtained from:

$$\varphi^{UF'} = \frac{C_u^{UF'}}{C_t^{UF}} \qquad \text{(Eq. 3)}$$

where $C_u^{UF'}$ and C_t^{UF} correspond to the apparent unbound concentration of ^{14}C-I in the filtrate and to the total concentration of ^{14}C-I in the spiked solution prior to filtration, respectively. The φ^{ED} value was similarly obtained from:

$$\varphi^{ED} = \frac{C_u^{ED}}{C_t^{ED}} \qquad \text{(Eq. 4)}$$

where C_u^{ED} and C_t^{ED} represent the albumin unbound concentration of ^{14}C-I in the dialysate and the total concentration of ^{14}C-I in the albumin solution after dialysis at equilibrium, respectively.

The mean value (±*SD*) for φ_{true} in four different experiments was 10.0 ± 0.53. Accordingly, the true unbound concentration of ^{14}C-I in the original albumin solution was 0.3 mg/ml, a value considerably smaller than the ^{14}C-I solubility in aqueous solution (≤1 mg/ml).

Based on these data, it was concluded that ^{14}C-I could be safely given by the intravenous route in a total concentration of 3.0 mg/ml in albumin (200 mg/ml).

Erythrocyte Partitioning of Proquazone[22]—The erythrocyte-

[22] The values of $P_{E/B}$ were corrected for binding of ^{14}C-I to hemoglobin in buffer. The hemoglobin resulted from slight hemolysis of erythrocytes suspended in buffer. The hemoglobin concentrations in buffer ranged between 0 and 25 mg/ml, and the percentage of ^{14}C-I bound to hemoglobin varied between 0 and 10%. The $P_{E/B}$ values were not normalized for pH 7.40.

Table IV—Erythrocyte–Buffer Partition Coefficient ($P_{E/B}$) and Erythrocyte–Plasma Partition Coefficient ($P_{E/P}$) of Proquazone in the Presence and Absence of Its Metahydroxy, Methylhydroxymetahydroxy, Methylhydroxy, and Carboxylic Acid Metabolites in Blood of Subject F

Total "Blood" Proquazone Concentration (C_{bl}), ng/ml	$P_{E/B}$, mean ± SD ($n = 4$)	$P_{E/P}$, mean ± SD ($n = 4$)
50	5.33 ± 0.172	0.086 ± 0.020
300	5.71 ± 0.351	0.089 ± 0.019
300[a]	5.60 ± 0.286	0.100 ± 0.018
2500	5.57 ± 0.183	0.094 ± 0.015
5000	5.53 ± 0.201	0.094 ± 0.018
Overall mean	5.55 ± 0.254	0.093 ± 0.017

[a] The concentration ratio, 20, is the sum of unlabeled metabolites to labeled proquazone.

buffer partition coefficient, $P_{E/B}$, was defined (30) as:

$$P_{E/B} = \frac{C_e}{C_u} \quad \text{(Eq. 5)}$$

where C_e and C_u correspond to the true erythrocyte and buffer (plasma water) concentrations of ^{14}C-I, respectively, after equilibration. Analogously, the erythrocyte–plasma partition coefficient, $P_{E/P}$, is:

$$P_{E/P} = \frac{C_e}{C_t} \quad \text{(Eq. 6)}$$

where C_t represents the total (bound and unbound) concentration of ^{14}C-I in plasma after equilibration.

In the centrifugation method, C_u or C_t was measured in buffer or plasma after centrifugal separation. The apparent erythrocyte concentration ($C_{e'}$) was measured in the remaining concentrated erythrocyte suspension. The hematocrit (Hc') of these suspensions was measured, and the true erythrocyte concentration of ^{14}C-I (C_e) was determined for the erythrocyte–buffer and erythrocyte–plasma suspensions, respectively, from:

$$C_e = \frac{C_{e'} - [(1 - Hc')C_u]}{Hc'} \quad \text{(Eq. 7a)}$$

$$C_e = \frac{C_{e'} - [(1 - Hc')C_t]}{Hc'} \quad \text{(Eq. 7b)}$$

The red blood cell partitioning of ^{14}C-I from buffer was large. The $P_{E/B}$ value averaged 5.5 (Tables IV and V), which is characteristic for lipophilic compounds (21, 31). The apparent red blood cell partitioning of ^{14}C-I from plasma was much smaller and averaged 0.09 (Table IV). The $P_{E/B}$ and $P_{E/P}$ values were not dependent on the concentration of ^{14}C-I within the range studied (50–50,000 ng/ml). There was good reproducibility of $P_{E/B}$ values in individual blood samples and red blood cell suspensions (Tables IV and V). The maximum percent interindividual difference in $P_{E/B}$ was 7% in the blood samples from the subjects (Table V). The red blood cell partitioning of ^{14}C-I was invariant in the presence of a large concentration ratio of total metabolites to parent drug (Tables IV and V). The extent of the red blood cell partitioning from buffer clearly depended on the pH of the suspensions. The $P_{E/B}$ value increased apparently linearly with increasing pH (Fig. 4) and was (n = 2) 4.72 (±0.100), 5.05 (±0.108), 5.40 (±0.219), and 5.80 (±0.176) at pH 6.62, 6.91, 7.23, and 7.53, respectively. This increase was equivalent to a 23% increase of the erythrocyte concentration of ^{14}C-I within the pH 6.62–7.53 range.

The $P_{E/B}$ value was invariant with time at the four pH levels studied (Fig. 4). Equilibration of ^{14}C-I between erythrocytes and buffer was rapid and completed in <2 min after addition of I at pH 7.4. The ratio of the erythrocyte concentration to the buffer concentration was time independent from 2 to 140 min after spiking, with the $P_{E/B}$ value remaining constant at 5.23 ± 0.142 (n = 9). This finding suggested that all partitioning values of ^{14}C-I obtained in these studies represented equilibrium values. A rapid attainment of equilibrium between red blood cells and buffer or plasma is consistent with the strongly lipophilic properties of ^{14}C-I (32). The large $P_{E/B}$ value of 5.5 found for ^{14}C-I indicated that there is an additional binding to, or greater solubility in, the red blood cells than can be accounted for by the assumption that the volume of the erythrocytes contained only an aqueous phase, wherein ^{14}C-I has the same chemical activity in buffer or plasma water. Red blood cell structures, which reportedly bind drug quantitatively, are hemoglobin (32), the membrane (33), and the enzyme carbonic anhydrase (34). The observed pH-dependent binding of ^{14}C-I to red blood cell structures is comparable to that to plasma proteins described earlier. Similar rationalizations may be given to explain both of these findings.

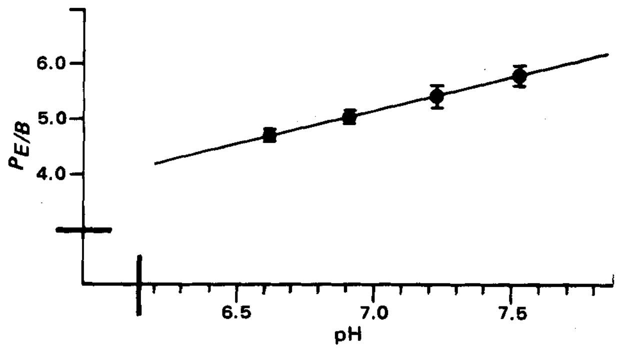

Figure 4—*Apparent pH dependency of the erythrocyte–buffer partition coefficient ($P_{E/B}$) of proquazone. Mean (±SD) (vertical bars) of four experiments performed at each PH level are given. Blood of Subject I was used.*

The results obtained for ^{14}C-I with proteins and erythrocytes indicate that studies of the binding kinetics of highly bound ligands may be useful in detecting and quantifying conformation and aggregation changes of extracellular and cellular macromolecules.

If only the plasma unbound drug partitioned into red blood cells, estimates of φ could be obtained from the erythrocyte partitioning method. The value of φ was defined in Eq. 1. If Eqs. 5 and 6 are solved for C_u and C_t, respectively, and the resulting expressions are substituted into Eqs. 7a and 7b, upon rearrangement Eq. 8 is obtained:

$$\varphi = 10^2 \left(\frac{P_{E/P}}{P_{E/B}}\right) \quad \text{(Eq. 8)}$$

and φ can be calculated directly from the erythrocyte–buffer and erythrocyte–plasma partition coefficients. The usefulness of the red cell partitioning method for protein binding determinations was demonstrated by the equivalent results obtained with this method and with equilibrium dialysis. On the average, constant φ values of 1.68 ± 0.255 (n = 20) and of 1.77 ± 0.026 (n = 15) were obtained with red cell partitioning and equilibrium dialysis, respectively, for the concentration range of 50–5000 ng of ^{14}C-I/ml. The close agreement of the binding data obtained with the two methods confirms that only unbound drug partitions into red blood cells. However, the precision of equilibrium dialysis was clearly greater than that of the partitioning method.

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Table V—Erythrocyte–Buffer Partition Coefficient of Proquazone ($P_{E/B}$) in the Presence and Absence of Its Metahydroxy, Methylhydroxymetahydroxy, Methylhydroxy, and Carboxylic Acid Metabolites in Blood of Subjects A–F

Total "Blood" Proquazone Concentration (C_{bl}), ng/ml	$P_{E/B}$, mean ± SD ($n = 4$)					
	Subject A	Subject B	Subject C	Subject D	Subject E	Subject F
300	5.34 ± 0.223	5.19 ± 0.079	5.76 ± 0.289	5.57 ± 0.415	5.80 ± 0.758	5.71 ± 0.351
300[a]	5.90 ± 0.340	—	—	—	—	5.59 ± 0.284
5000	5.31 ± 0.537	5.25 ± 0.118	5.53 ± 0.346	4.96 ± 0.279	5.25 ± 0.556	5.23 ± 0.201
Overall mean ± SD	5.51 ± 0.493	5.22 ± 0.998	5.64 ± 0.320	5.26 ± 0.460	5.52 ± 0.681	5.63 ± 0.274

[a] The concentration ratio, 20, is the sum of unlabeled metabolites to labeled proquazone.

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Simultaneous Determination of Imipramine, Desipramine, and Their 2-Hydroxy Metabolites in Plasma by Ion-Pair Reversed-Phase High-Performance Liquid Chromatography with Amperometric Detection

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Abstract □ An ion-pair reversed-phase high-performance liquid chromatographic (HPLC) method, using an electrochemical detector, is presented for the simultaneous and rapid quantitation of imipramine, desipramine, and their 2-hydroxylated metabolites in plasma. The drugs are extracted from 1 ml of plasma at pH 9.7 with ether, back-extracted into 0.1 *M* HCl, and reextracted into ether following alkalinization. An efficient electrochemical oxidation reaction at the detector electrode affords a low detection level of ~5 ng/ml in a mobile phase of acetonitrile–acetate buffer (40:60) containing 0.005 *M* heptanesulfonate. Patient data are presented as correlations between the plasma level of each hydroxy metabolite and its respective parent compound. The method is applicable to the laboratory experienced in HPLC.

Keyphrases □ Imipramine—simultaneous determination with desipramine and their 2-hydroxy metabolites, ion-pair reversed-phase high-performance liquid chromatography □ Desipramine—simultaneous determination with imipramine and their 2-hydroxy metabolites, ion-pair reversed-phase high-performance liquid chromatography □ High-performance liquid chromatography—simultaneous determination of imipramine, desipramine, and their 2-hydroxy metabolites

Considerable interest exists in the relationship between the plasma concentration of tricyclic antidepressant drugs and the therapeutic outcome or side effects, and this subject was reviewed recently (1, 2). There seems to be a consensus that there is a therapeutic range for nortriptyline and a minimum effective level for imipramine plus desipramine in patients with "endogenous-type depression"; a therapeutic range also was suggested for desipramine (3). Data for amitriptyline, however, are much more controversial (4), and other tricyclic antidepressants have not been studied adequately.

BACKGROUND

Until recently, the role of the hydroxylated metabolites of the tricyclic antidepressant drugs has been largely ignored, because it was assumed (erroneously) that these metabolites were not psychoactive, did not cross the blood–brain barrier, and were rapidly excreted. Christianssen and Gram (5) demonstrated that these hydroxylated metabolites were present in the central nervous system in an acute overdose case. Several studies demonstrated considerable quantities of unconjugated and conjugated hydroxy metabolites of tricyclic antidepressants in the plasma of treated patients (6–11).

It is now known that the hydroxy metabolites of imipramine have strong cardiovascular activity (12, 13) and are essentially equipotent to the parent compound in the blockade of norepinephrine and 5-hydrox-

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