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Report

Binding of Pyrimethamine to Human Plasma Proteins and Erythrocytes

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A high-performance liquid chromatographic (HPLC) assay was developed for pyrimethamine in plasma, red blood cells (RBCs), and buffer for the purpose of studying its plasma protein binding and RBC partitioning. Pyrimethamine (1000 ng/ml) was 94% bound to plasma proteins on average, depending on the pH of plasma. A comparison of the lower and upper range of plasma concentrations that would be achieved after a malaria prophylaxis dosing regimen (25 mg/week) showed that the fraction unbound was significantly lower at 120 ng/ml than at the upper plasma concentration of 360 ng/ml, 3.5 vs 4.9%, respectively. Nonlinear regression of the effect of albumin concentration (g/L) on plasma binding yielded the equation: fraction unbound = $1/[(0.421 * \text{albumin concentration}) + 1]$ ($R^2 = 0.99$). There was no binding to normal levels of alpha$_1$-acid glycoprotein (AAG). The mean ratio of the concentration of pyrimethamine in RBCs to that in plasma (RBC:plasma ratio) was 0.42, while the mean RBC:buffer ratio was 5.2. Binding to hemolysate did not account for all of the RBC uptake, suggesting that binding to or partitioning into RBC membranes may be important. Because pyrimethamine binding depends on both albumin concentration and pyrimethamine concentration in the plasma, these studies predict greater free fractions of pyrimethamine associated with the higher doses given for toxoplasmosis (75 mg/day) and with the hypoalbuminemia associated with AIDS and malaria.

KEY WORDS: pyrimethamine; malaria; toxoplasmosis; protein binding; erythrocytes; pyrimethamine assay.

INTRODUCTION

Pyrimethamine is a dihydrofolate reductase inhibitor that has been used in the prophylaxis and suppression of malaria since the 1950s (1) and is presently, in combination with sulfadiazine or trisulfapyrimidines, the mainstay of treatment of toxoplasmosis (2,3). Recently, the use of pyrimethamine has greatly increased because of the high incidence of toxoplasma encephalitis in patients with AIDS (2). In addition, the doses used for toxoplasmosis are much higher than those used for malaria and the resulting plasma concentrations have proven to be unpredictable (2). For malaria prophylaxis, the usual dose is quite small, 25 mg *per week* (4). However, for toxoplasmosis in immunodeficient patients, the recommended loading dose for adults is 100–200 mg, followed by a maintenance dose of 75–100 mg *per day* (5). Accordingly, greatly increased plasma concentrations will result from the higher doses given for toxoplasmosis.

Although pyrimethamine has been widely used for malaria for many years, a review of the literature on pyrimethamine indicates that the significance of its erythrocyte uptake has not been appreciated. The plasmodia spends most of its growth phase inside red cells (6), suggesting that RBC uptake into the cell water would be important for antimalarial activity and the study of the pharmacodynamics of the drug in malaria.

Pyrimethamine is a highly protein-bound, lipophilic weak base with a pK_a of 7.13 (7) and an elimination half-life of 85 hr in healthy male volunteers (8). In order better to understand pyrimethamine distribution in the blood, our objectives were to characterize its plasma protein binding and RBC partitioning and binding and to examine the relationship between the plasma and the RBC concentrations of pyrimethamine.

MATERIALS AND METHODS

Materials

Pyrimethamine [5-(4-chlorophenyl)-2,4-diamino-6-ethylpyrimidine] base was a gift of Dr. Carl Sigel and the Burroughs Wellcome Co. (Research Triangle Park, NC). *N*-Acetyl-procainamide (NAPA) HCl (Aldrich Chemical Co., Milwaukee, WI) was used as the internal standard. HPLC-grade monobasic potassium phosphate and reagent-grade dibasic potassium phosphate (J. T. Baker Chemical, Phillipsburg, NJ) were used for the mobile phase buffer. All

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other phosphate buffers were ACS grade (Fisher Scientific Co., Fair Lawn, NJ).

Extraction, Apparatus, and Chromatographic Conditions

After the addition of 100 μl of a 0.8 μg/ml solution of NAPA in methanol, pyrimethamine and NAPA were extracted from 0.5 ml of plasma using 7 ml of methylene chloride by shaking for 10 min. Because of the excessive variability in the extraction of NAPA from RBCs, pyrimethamine was extracted from 0.5 ml RBCs quantitatively, without the internal standard, using a 1:3 mixture (12 ml) of methylene chloride and *n*-butyl chloride by shaking for 20 min. Aliquots (100 μl) of buffer from dialysis experiments were injected directly onto the high-performance liquid chromatograph (HPLC) in order to determine pyrimethamine concentrations.

The HPLC consisted of a Model 110 A Altex pump (Beckman Instruments, Berkeley, CA), a Model 440 UV detector at 254 nm at a sensitivity of 0.005 or 0.01 aufs (Waters Associates, Milford, MA), a syringe-loading injector with a 100-μl loop (Model 7125, Rheodyne, Berkeley, CA), a 2-cm × 2-cm-i.d. Uptight precolumn packed with Perisorb RP-18 30- to 40-μm pellicular packing (Upchurch Scientific, Oak Harbor, WA), and a 15-cm × 4.6-mm-i.d. Econosphere C18 column containing 5-μm octadecylsilane packing material (Alltech Associates/Applied Science Labs, Deerfield, IL). The mobile phase consisted of 45% methanol, 15% acetonitrile, and 40% potassium phosphate buffer (pH 6.95, 0.03 *M*) at a flow rate of 1.6 ml/min. Under these conditions pyrimethamine eluted at 5.35 min, and NAPA at 4.5 min. Linear calibration curves were constructed separately over two ranges for each matrix (plasma, buffer, and RBCs) for pyrimethamine concentration ranges 24–140 and 140–1000 ng/ml. Quantification was accomplished by means of the peak height ratio of pyrimethamine/NAPA for plasma and peak heights of pyrimethamine for RBCs and buffer. The limit of detection was 24 ng/ml for each of the matrices. Interday and intraday coefficients of variation for controls and standards were less than 8% in all matrices. Interday coefficients of variation for the lowest calibration concentration (24 ng/ml) were 3.1% ($n = 6$), 3.6% ($n = 9$), and 7.1% ($n = 10$) for plasma, RBCs, and buffer, respectively. Validation of this assay was presented previously (9,10).

Binding of Pyrimethamine to Albumin, Plasma Proteins, and Alpha$_1$-Acid Glycoprotein (AAG)

The protein binding of pyrimethamine was determined by equilibrium dialysis using a Spectrum dialyzer (Spectrum Industries, Los Angeles, CA) with polytetrafluoroethylene cells (1 ml/cell-half) and 6000–8000 molecular weight cutoff dialysis membranes (Spectrapor 1 cellulose tubing, Spectrum Industries). Unless otherwise noted isotonic phosphate buffer at pH 7.4 was dialyzed against plasma. The time necessary to reach equilibrium was determined in preliminary experiments. In general, dialysis was performed for 5 hr in a 37°C water bath and both plasma and buffer in each cell were assayed for pyrimethamine. Binding to dialysis cells and membranes was evaluated. Recovery of pyrimethamine was assessed by dialyzing replicate samples of 85 ng/ml pyrimethamine in buffer against buffer ($n = 5$). No binding to dialysis cells or membranes was found.

A sample from each cell-half was assayed for albumin content at the end of each dialysis. The albumin measurement was used to provide assurance of identical conditions in each cell. The absence of protein in the buffer side indicated membrane integrity. Albumin concentrations before and after dialysis were compared to evaluate volume shifts (11). Albumin was assayed using BCG albumin reagent (Stanbio Laboratory, Inc., San Antonio, TX) and a spectrophotometric assay including a three-point calibration curve using albumin standards in normal saline (Sigma Diagnostics, St. Louis, MO). The fraction unbound of pyrimethamine in plasma was calculated by dividing the pyrimethamine concentration in the buffer cell-half (unbound drug) by the pyrimethamine concentration in the corresponding plasma cell-half (total drug) after equilibrium had been reached. Total pyrimethamine concentrations reported are the plasma pyrimethamine concentrations at the end of dialysis. By assaying plasma and buffer, any drug loss due to transferring by pipette or otherwise was taken into account.

Similarly, the influence of plasma pH on binding was examined. Solutions of isotonic phosphate buffer at pH 6.3, 6.5, 6.7, 6.9, 7.0, 7.1, 7.7, and 8.0 were prepared. These buffer solutions were spiked with pyrimethamine (400 ng/ml) and dialyzed against plasma with the same pH in triplicate. As in all experiments, plasma pH was adjusted immediately before loading the dialysis cells by blowing 5% CO_2/95% O_2 over the plasma (12). Plasma pH was adjusted to about 0.1 unit below the desired ending pH because the pH rose about 0.1 unit during dialysis. The pH values reported are at the end of dialysis.

To examine the influence of anticoagulants on plasma protein binding, blood was collected from drug-free volunteers into three kinds of Vacutainers (Becton-Dickinson, Rutherford, NJ): Vacutainers containing (1) EDTA or (2) heparin for plasma and (3) additive-free (red-top) Vacutainers for serum. Isotonic phosphate buffer solutions spiked with pyrimethamine (1200 ng/ml) were dialyzed against plasma or serum in triplicate at pH 7.4.

The concentration dependence of pyrimethamine protein binding was determined by dialyzing heparinized plasma against pH 7.4 isotonic phosphate buffer containing pyrimethamine at two concentrations. Total plasma concentrations of pyrimethamine at the end of dialysis were 120 ng/ml ($n = 5$) and 360 ng/ml ($n = 5$). Albumin concentration was 46 g/liter.

Binding to pure albumin (Pierce Product No. 30445 affinity-purified human serum albumin, Pierce Chemical Co., Rockford, IL) and human AAG (99% purity, Sigma Chemical Co., St. Louis, MO) was evaluated by dialyzing isotonic phosphate buffer solutions of pyrimethamine (350 ng/ml) against buffer solutions of albumin (14.2 g/liter) and AAG (0.6 and 3.7 g/liter) for 5 hr.

Three studies were performed for determining plasma protein binding constants. Blood was collected by venipuncture from drug-free volunteers into Vacutainers containing heparin. The blood was centrifuged and the plasma was removed. Plasma albumin concentration was adjusted by adding isotonic buffer before dialysis. In study 1, plasma was dialyzed against isotonic buffer containing pyrimethamine at

140, 280, 480, 720, 1440, and 4100 ng/ml in duplicate. Albumin was 23.5 g/liter (3.4×10^{-4} *M*) at the end of dialysis. In study 2, heparinized plasma was dialyzed against buffer containing pyrimethamine at 140, 250, 400, 600, 780, 1200, 2200, and 4100 ng/ml in duplicate. Albumin was 8.13 g/liter (1.2×10^{-4} *M*) at the end of dialysis. In study 3, heparinized plasma was dialyzed against buffer containing pyrimethamine at 720, 3700, 9100, 18,000, and 45,000 ng/ml in duplicate. Albumin was 0.66 g/liter (9.6×10^{-6} *M*). Using SAS NLIN (13), the data collected were fit to nonlinear stoichiometric equations assuming one or two binding constants (14) and using the reciprocal of the free drug concentration as the weighting factor.

Data were fit to nonlinear stoichiometric equations with one and two binding constants. The best fits were obtained with the two-constant model shown below:

$$R = \frac{K_1[D] + 2K_1K_2[D]^2}{1 + K_1[D] + K_1K_2[D]^2} \quad (1)$$

where R equals the number of moles of drug bound per mole of total protein, $[D]$ is the unbound drug concentration, K_1 is the affinity constant for the binding of the first molecule of drug to protein, and K_2 is the affinity constant for the binding of the second molecule of drug to the protein molecule. When the amount of bound ligand is small compared to that of the protein and the binding is not strongly cooperative, the first stoichiometric binding constant can be estimated from this simplification of Eq. (1) (15):

$$K_1 = ([D_b]/[D])/[\text{albumin}] \quad (2)$$

where $[D_b]$ is the bound drug concentration and [albumin] is the plasma albumin concentration.

Partitioning of Pyrimethamine into RBCs

For examining binding to hemoglobin, blood was obtained from a drug-free subject and transferred by syringe into four Vacutainers containing heparin. After centrifugation, the plasma and buffy coat were removed. The RBCs were washed three times with isotonic phosphate buffer. An approximately equal volume of distilled-deionized water was added to the packed cells. The tubes were vortexed 2 min and complete hemolysis was assumed. Membrane-free hemolysate was obtained by centrifugation in a Sorvall RC-58 Superspeed centrifuge (DuPont Co. Instrument Products, Newtown, CT) at 27,000 × *g* for 30 min at room temperature. The hemolysate was decanted for further analysis and the pellet was discarded. The hemoglobin concentration was measured using the Sigma Diagnostics Total HB kit (Proc. No. 525, Sigma Chemical Co., St. Louis, MO). The binding of four concentrations of pyrimethamine (45, 190, 250, and 590 ng/ml of hemolysate after dialysis) to hemolysate was investigated by dialysis against pH 7.4 isotonic phosphate buffer for 5 hr.

Preliminary studies showed that equilibrium between RBCs and buffer or plasma was reached very quickly. The ratio of the concentration of pyrimethamine in RBCs to that in plasma (RBC:plasma ratio) was determined by adding whole blood to tubes in which 2–3 μg of pyrimethamine had been placed and rocking them for 20 min. The tubes were centrifuged and pyrimethamine was measured in the plasma and in the packed RBCs.

The ratios of the concentration of pyrimethamine in RBCs to that in buffer (RBC:buffer ratios) were determined by adding washed RBCs to buffer (spiked with pyrimethamine) in plastic vials and rocking for 20 min. The concentrations of pyrimethamine were determined in aliquots of both the RBCs and buffer. The RBC concentration of pyrimethamine was corrected for trapped buffer using the hematocrit of the RBC suspension and the concentration of the drug in the buffer (16).

The measured RBC:buffer ratios were compared to the theoretical ratios for unbound pyrimethamine in RBCs and medium. The theoretical ratio is based on the pK_a of the drug (7.13) and the pH of the cell water (7.26) (17) and the medium. The equation describing the relationship between cell and medium concentrations of a weak base is (18):

$$\frac{C_{cell}}{C_{medium}} = \frac{1 + 10^{(pK_a - pH_{cell})}}{1 + 10^{(pK_a - pH_{medium})}} \quad (3)$$

A difference between the measured and the theoretical cell:medium ratios would indicate binding to the proteins in the hemolysate (cell contents, mainly hemoglobin) or the cell membrane.

The effect of the buffer pH on the RBC:buffer ratio was evaluated by making six 400 ng/ml solutions of pyrimethamine in isotonic phosphate buffer at pH 6.7, 7.1, 7.4, 7.7, 7.9, and 8.0 and adding packed RBCs to them in plastic vials. After rocking for 20 min, aliquots of buffer and packed cells were assayed for pyrimethamine.

To determine the dependence of the partitioning of pyrimethamine into RBCs upon plasma albumin levels, pyrimethamine was evaporated from methanol in silanized culture tubes. Mixtures of buffer, plasma, and washed RBCs were added to the tubes to get four different dilutions of plasma proteins at the same hematocrit. The RBCs and the plasma buffer were assayed after equilibration for pyrimethamine. The pyrimethamine concentration of the RBCs was corrected for trapped plasma.

Statistical analyses were performed using SAS ANOVA, GLM, REG, and TTEST procedures (13).

RESULTS

There was a significant difference ($P < 0.03$) in the unbound fractions at two concentrations of pyrimethamine within the antimalarial therapeutic range (21) at a plasma albumin level of 45.6 g/liter. The mean ± SD percentage unbound at 120 ng/ml was 3.5 ± 0.50 and that at 360 ng/ml was 4.9 ± 0.98. Because of assay limits (24 ng/ml) for the free drug concentration, lower concentrations could not be studied.

There was no difference in the fraction unbound in plasma containing anticoagulants (EDTA or heparin) or serum when the samples were adjusted to the same pH. The percentage unbound pyrimethamine (990 ng/ml) was 6.1 ± 1.2 ($n = 9$) with albumin, 41.8 g/liter at pH 7.4. Disregarding dilution from the minute water content of the membranes, there were only small volume shifts. The mean ± SD percentage reduction in albumin concentration was 4.2 ± 5.7 (*n*

= 69) for 5-hr dialyses. Corrections for volume shifts were not made.

The influence of pH on plasma binding is shown in Table I. The mean percentage unbound (total plasma pyrimethamine concentration, 360 ng/ml) at pH 7.4 was 8.8 ± 1.6 and that at pH 7.93 was 5.8 ± 0.64. The differences in binding at pH 7.4 versus pH 7.93 did not reach statistical significance, but there is a trend for increased binding at higher pH values.

Using data from the studies for the determination of binding constants in which whole plasma had been used, nonlinear regression of the effect of albumin concentration (g/liter) on protein binding yielded the equation, fraction unbound = 1/[(0.421 * [albumin]) + 1] (R^2 = 0.99) and is illustrated in Fig. 1. This equation can be derived from the law of mass action by making the following assumptions: a small number of binding sites are occupied, there is only one class of binding sites (hence only one affinity constant), and the dissociation constant K_d is much larger than the free drug concentration. In this case, the pyrimethamine concentration was 1.4 μM; K_d (the reciprocal of the affinity constant K_1 from Table II) was 35 μM; the albumin concentration varied between 120 and 660 μM; and over the range of albumin concentrations in Fig. 1, only one class of binding sites was evident (Table II, study 1), suggesting that the assumptions for the above equation are reasonable.

The percentage bound to pure albumin (14.2 g/liter isotonic phosphate buffer) was 86.5 ± 0.33 at a pyrimethamine concentration of 350 ng/ml. This agreed with the 85.5% predicted by the equation above for fraction unbound at an albumin concentration of 14.2 g/liter, suggesting that albumin accounts for most, if not all, of the binding in plasma. Pyrimethamine binding was determined at two levels of pure AAG in pH 7.4 isotonic buffer. At an AAG level of 3.7 g/liter buffer, pyrimethamine was 9.6% bound. At a normal level of AAG (0.6 g/liter buffer), no binding was apparent.

Results of fits to data collected in three studies for the determination of binding constants are presented in Table II. The best fit for study 2 is shown in Fig. 2. The best fits were obtained to the model with two binding constants [Eq. (1)] for studies 2 and 3, but for study 1 a one-constant model fits equally well, probably because the higher-affinity binding was not revealed until the albumin concentration was lowered. The second affinity constant for study 2 probably reflects nonspecific binding. The fraction unbound varied only from 7.4 to 9.6% (mean, 8.7 ± 0.7%) throughout the range of pyrimethamine concentrations in study 1 (140–4100 ng/ml). Equation (2) gave very similar results for K_1 compared to Eq. (1).

Table I. Influence of pH on Fraction Unbound in Plasma

pH	Fraction Unbound (×100), mean (±SD)
6.75	13.9 (1.02)
6.82	14.6 (1.36)
6.97	14.7 (0.68)
6.98	11.7 (2.47)
7.15	10.1 (0.14)
7.4	8.8 (1.58)
7.7	7.3 (1.19)
7.93	5.8 (0.64)

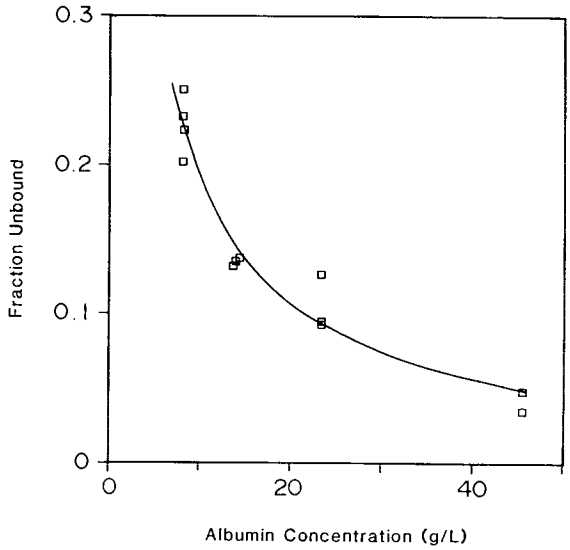

Fig. 1. Relationship between the fraction unbound of pyrimethamine (360 ng/ml) and the plasma concentration of albumin at 37°C. The solid line represents the line of best fit.

Overall, partitioning into RBCs decreased as plasma albumin increased. For plasma pyrimethamine concentrations of 980 ng/ml (albumin, 44.8 g/liter), the RBC:plasma pyrimethamine concentration ratio was 0.42 ± 0.043 (n = 5) at a normal hematocrit of 0.43.

The partitioning of pyrimethamine into RBCs was affected by the pH of the buffer in which the RBCs were suspended. The ratio of pyrimethamine concentration in RBCs to that in protein-free buffer (RBC:buffer ratio) increased over the range of buffer pH 6.7 to 8, where the ratio increased from 3.8 to 6.9.

Drug associated with RBCs may be (1) bound to the cell membrane, (2) bound to intracellular proteins, of which the most prominent is hemoglobin, or (3) partitioned into the cell water. At buffer pH 7.4, the RBC:buffer ratio was 5.16 ± 0.61 (n = 2). The theoretical cell:medium ratio is 1.13 [Eq. (3) assuming cell water, pH 7.26, and medium, pH 7.4]. In other words, there was 4.6 times as much pyrimethamine in the RBCs as predicted by the theoretical ratio. The mean percentage bound to hemolysate (hemoglobin, 1.6 × 10^{-3} M) was 42.5 ± 8.1. Since binding to hemoglobin does not appear to account for all of the drug associated with the RBCs, these results suggest additional binding to or partitioning into the cell membrane.

DISCUSSION

Different values for stoichiometric binding constants were found using plasma samples with varying albumin concentrations. This phenomenon may be explained by the masking of sites as a result of the aggregation of protein molecules at higher concentrations (19). Dilution of plasma may result in the uncovering of higher affinity binding sites (20). Estimates of the affinity constants using Eq. (2) were in excellent agreement with estimates from nonlinear regression fits to Eq. (1) (Table II). These results support the use of more than one albumin concentration in binding studies for determining binding affinity constants.

Table II. Summary of Apparent Binding Constants

Study	Albumin (g/L)	Eq. (2) Estimate K_1 (M^{-1})[a]	NLIN Estimate[b] K_1 (M^{-1})	NLIN Estimate[b] K_2 (M^{-1})
1	23.5	29,100	32,000	None
		(4,550)	(1,170)	
2	8.1	28,400	28,200	17,400
		(2,460)	(873)	(8,250)
3	0.7	51,700	53,200	24,900
		(16,700)	(7,210)	(8,120)

[a] Estimated from Eq. (2), mean (±SD).
[b] Estimated by SAS NLIN fit to Eq. (1), mean (±asymptotic SE).

For malaria prophylaxis, the usual dose of 25 mg/week produced an average maximum plasma pyrimethamine concentration of 314 ng/ml (21). However, for toxoplasmosis in immunodeficient patients, one study reported pyrimethamine serum concentrations of 1333–4472 ng/ml after a 50 mg/day dosing schedule (2). We found a significant difference in binding at two different pyrimethamine concentrations (120 and 360 ng/ml) in the therapeutic range following an antimalarial dose of 25 mg. Because of this saturable binding, the higher doses given for toxoplasmosis should produce higher unbound fractions of pyrimethamine. However, in study 1 unbound fractions at 4100 ng/ml were similar to that at 350 ng/ml, 8.4 vs 9.6%, respectively, when a lower albumin concentration was used. Therefore, clinically, small differences in the fraction unbound because of saturable binding will not be as important as the changes in binding seen with different plasma concentrations of albumin. Figure 1 shows the pronounced increase in fraction unbound at albumin concentrations below 20 g/liter. Hypoalbuminemia is common in patients with malaria (22) and AIDS (23,24), and our results suggest large increases in the fraction unbound in cases of hypoalbuminemia. Clinically, by examining a patient's albumin concentration and the relationship between albumin concentration and fraction unbound, it may be possible to estimate the degree to which binding is altered.

Our results indicate that the major determinants of pyrimethamine binding in blood are albumin and, to a lesser extent, RBCs. In the therapeutic range of pyrimethamine concentrations in plasma, the protein binding is nonlinear, and therefore, because RBC uptake depends on the protein binding in the plasma, the relationship between RBC and plasma pyrimethamine concentration is nonlinear. RBC binding did not appear to be saturable and an altered hematocrit would not be expected to change plasma concentrations.

The high frequency of adverse reactions to treatment is a major problem in the treatment of CNS toxoplasmosis. Seventy-one percent of patients treated with pyrimethamine and sulfadiazine developed side effects in one study (3). If the intrinsic clearance of pyrimethamine is lowered by AIDS, higher free concentrations may be produced. Further studies are needed to determine if the intrinsic clearance is changed by AIDS and if the free concentrations as well as the unbound fractions of pyrimethamine are altered by hypoalbuminemia and AIDS.

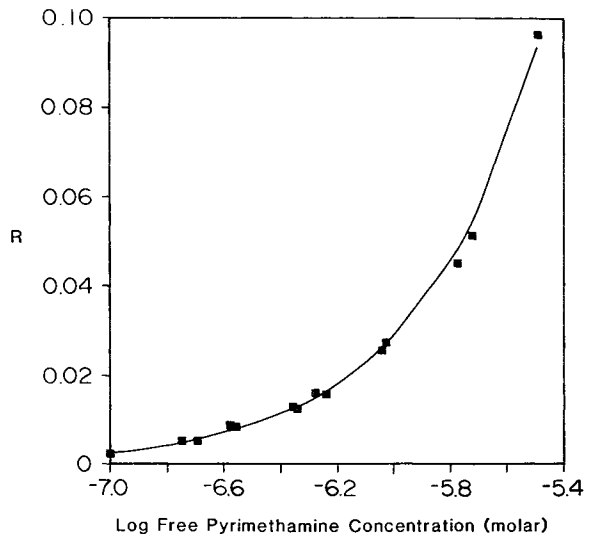

Fig. 2. The curve representing the best fit of the stoichiometric binding equation [Eq (1)] to data collected in study 2. The observations are from the same study.

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