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Pharmacokinetics of Ibuprofen in Man—III: Plasma Protein Binding

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Plasma protein binding of ibuprofen was measured by equilibrium dialysis on 406 plasma samples collected from 15 normal volunteers following doses of 400, 800, and 1200 mg of ibuprofen as tablets (N = 102, 100, 104, respectively) and 420 mg as an aqueous solution (N = 100). Individual subject bound concentration at dialysis equilibrium (C_{bd}) vs. free concentration at dialysis equilibrium (C_{fd}) were well fitted via computer to the Scatchard equation with one class of binding sites. The binding capacity averaged 1231 μM (range 848–1658 μM), and the association constant averaged $1.76 \times 10^5\ M^{-1}$ (range 1.15×10^5 to $2.73 \times 10^5\ M^{-1}$). Distributional analysis was performed on the free fraction (f_d) and bound/free ratios ($C_{bd}/C_{fd} = 1/f_d - 1$) at dialysis equilibrium for each treatment. Using pooled data of all four treatments, distributional analysis was also performed on the free fractions (f) and bound/free ratios ($C_b/C_f = 1/f - 1$) corresponding to the plasma drug concentrations in blood as it was withdrawn from the subjects. The bound/free ratios were normally distributed, whereas the distributions of the free fractions were skewed towards higher values.

KEY WORDS: ibuprofen plasma protein binding; bound/free ratio normally distributed; free fraction not normally distributed; binding capacity; association constant.

INTRODUCTION

Ibuprofen, 2-(4-isobutylphenyl)-propionic acid, is widely used in the treatment of rheumatoid arthritis and osteoarthroses. Mills *et al.* (1) reported that ibuprofen at a concentration of 20 μg/ml was 99% bound in whole human plasma. Whitlam *et al.* (2) and Kober and Sjöholm (3) studied the binding of ibuprofen to human serum albumin by the equilibrium dialysis

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method, while Whitlam and Brown (4) reported data on the binding of ibuprofen to 4% bovine serum albumin, 1% human serum albumin, and one sample of whole human serum by the ultrafiltration method. Wanwimolruk *et al.* (5) reported postdialysis serum free fraction for ibuprofen at a total concentration of 40 mg/L for 10 patients with rheumatoid arthritis and 10 patients with osteoarthritis.

The human study discussed in this series of articles was designed to elucidate the cause of the nonlinear relationship between area under the total (bound+free) ibuprofen plasma concentration-time curve and the administered dose of the drug. It was found that the area under the free (unbound) plasma concentration-time curve was a linear function of dose, and hence the nonlinearity in the case of total drug was attributed to the nonlinear plasma protein binding. The area-dose relationships are reported in another article (6). We report here in detail the results of the plasma protein binding studies and thus provide a considerable amount of data on the plasma protein binding of ibuprofen in the presence of its metabolites and in whole human plasma under conditions of use of the drug in man.

EXPERIMENTAL PROCEDURE

Human Study

Fifteen healthy nonobese male volunteers with no known disease were selected. The average and range were: 25 (22–35) years, 78.2 (71.7–92.5) kg, and 2.01 (1.89–2.24) m^2 body surface area. Normal complete physical examination, routine blood and urinalysis, and normal values for kidney and liver function tests were necessary for entry into the study. Informed consent was obtained from each subject. All subjects participating in the study received no barbiturates or other enzyme-inducing agents for a period of 30 days preceding initiation of the study and none concurrent with it. They received no other medication or alcoholic beverages for a period of 7 days before initiation of the study and none during the study.

Treatments A, B, and C were as follows: A, One 400 mg ibuprofen tablet[4]; B, two 400 mg ibuprofen tablets; C, three 400 mg ibuprofen tablets. The tablets were assayed at 401 mg/tablet; hence doses were 401, 802, and 1203 mg ibuprofen for treatments A, B, and C, respectively. Treatment D was 20 ml of an oral solution of ibuprofen, 20 mg/ml; the solution was assayed at 21.0 mg/ml; hence the dose was 420 mg of ibuprofen. The treatment schedule is shown in Table I. Subjects received treatments A, B, and C in crossover fashion in weeks 1 to 3; then all subjects received

[4]Courtesy of C. C. T. Motrin (The Upjohn Company).

Table I. Treatment Schedule in Human Study

Group	Subjects/group	Treatment in indicated phase			
		I	II	III	IV
1	2, 6, 8, 11, 15	*A*	*B*	*C*	*D*
2	1, 3, 9, 13, 14	*B*	*C*	*A*	*D*
3	4, 5, 7, 10, 12	*C*	*A*	*B*	*D*

treatment D in the fourth week. Treatments were separated by a one-week period.

Subjects fasted overnight (from 10 p.m.) and for 4 hr after dosing. For treatments A, B, and C, 5 ml of blood was collected by venipuncture at 0, 0.167, 0.333, 3, 10, and 12 hr, and 10 ml was collected at 0.5, 1, 1.5, 2, 4, 6, and 8 hr. For treatment D, 5 ml of blood was collected at 0, 0.0833, 0.167, 0.25, 0.333, and 3 hr, and 10 ml was collected at 0.5, 1, 1.5, 2, 4, 6, and 8 hr.

Assay of Plasma and Plasma Protein Binding

Aliquots of all plasma samples were assayed for unchanged ibuprofen by a sensitive and specific HPLC method (7). Aliquots of plasma obtained from the 10 ml blood samples were used in plasma protein binding studies. A 1 ml volume of plasma was dialyzed against a 3 ml volume of phosphate buffer (0.693 g dibasic potassium phosphate, 0.138 g monobasic sodium phosphate, 2.25 g sodium chloride, made up to 500 ml with water then adjusted to *p*H 7.4). The dialysis membrane was Spectrapor® 2 tubing (Spectrum Medical Industries, Los Angeles, Calif.) having a flat width of 10 mm. The tubing was cut into lengths of 15 cm, soaked in water for 10 min, and then in methanol for an additional 10 min. Following the methanol soak the membrane was thoroughly washed with distilled water (5 changes) and was then soaked in the dialysis buffer for at least 1 hr before use. Prior to dialysis the tubing was removed from the buffer, and excess buffer was gently wiped off. One end of the tube was firmly tied with string, then 1 ml of plasma spiked with a suitable amount of radioactive ibuprofen was carefully introduced into the open end. The open end was tied with string, and the tube was folded in a U-shape and placed inside a 1.5×10 cm test tube containing 3 ml of phosphate buffer. The tops of the tubes were sealed with parafilm to minimize buffer loss due to evaporation. The tubes were placed in racks in a light-tight shaking water bath set at 37°C. Dialysis was allowed to proceed for 8 hr, shown to be the time necessary for equilibration. At the end of the dialysis period the tubing was removed from the buffer, gently dried on the outside, then inverted 10 times to ensure adequate mixing of the contents. One end of the tubing was cut, and a 100 μl aliquot of the

dialyzed plasma was added to 15 ml of ACS scintillation fluid (American Corp., Arlington Heights, Ill.) along with 900 μl of water. To a scintillation vial containing the same scintillation cocktail was added 2 ml of the buffer from the same dialysis tube. The contents of the vials were thoroughly mixed and were counted for 10 min in a Beckman LS7500 Scintillation Counter (Beckman Instruments, Fullerton, Calif.). The counts obtained were corrected for background and quenching effects.

The radioactive ibuprofen was 2-(4-isobutylphenyl)-[3-^{14}C]propionic acid with a specific activity of 22.6 μCi mg^{-1} and was radiochemically pure. A stock solution was prepared such that when 10 μl of this solution was added to 1 ml of plasma, the counts obtained were in the region of 50,000 dpm/ml.

For calculating the free (C_{fd})[5] and bound (C_{bd}) drug concentrations at dialysis equilibrium, the method outlined recently by Tozer (8) was employed. This method is independent of volume changes that may occur in dialysis systems due to the osmotic movement of water (9). The free fraction at dialysis equilibrium, f_d, was obtained as the ratio $C_{fd}/(C_{fd}+C_{bd})$.

Since drug is lost from the protein compartment during dialysis, the values of f_d and the total drug concentration ($C_{td}=C_{fd}+C_{bd}$) do not correspond to the values of the original plasma sample before dialysis. Correction back to this original C_t has been discussed previously by Behm and Wagner (10). In this study the estimation of the free (C_f) and bound (C_b) drug concentrations corresponding to C_t was carried out as follows.

Pooled C_{fd}, C_{bd} data for each subject (25–28 data points, 5–7 data points from each of four phases) were computer-fitted to the Scatchard equation (11) with one class of binding site:

$$C_{bd}=\frac{P(1)C_{fd}}{P(2)+C_{fd}} \tag{1}$$

Fitting was performed using the weighted least squares linear regression method and refinement outlined by Wilkinson (12) on an Apple II microcomputer. For all subjects the fits were excellent with coefficients of determination averaging 0.988 (range 0.942–0.997). This method has been shown by us to yield the same results as obtained by nonlinear least squares fitting of data to Eq. (1). Knowing

$$C_t=C_f+C_b \tag{2}$$

and substituting Eq. (1) into (2) to give

$$C_t=C_f+\frac{P(1)C_f}{P(2)+C_f} \tag{3}$$

[5]See Appendix for definition of symbols.

enables one to rearrange Eq. (3) into a quadratic equation of the form

$$C_f^2 + [P(1) + P(2) - C_t]C_f - p(2)C_t = 0 \tag{4}$$

Thus, knowing the values of $P(1)$ and $P(2)$ for any subject enables you to solve Eq. (4) at any desired C_t and obtain a value of C_f as the positive root of the equation. C_b is then simply found as the difference between C_t and C_f.

The bound/free ratio, C_b/C_f, is related to the free fraction, $f = C_f/(C_f + C_b)$, by Eq. (5):

$$\frac{C_b}{C_f} = \frac{1}{f} - 1 \tag{5}$$

Five sets of binding data were evaluated to determine whether the bound/free ratios or the free fractions were normally distributed. Statistical analysis was performed using the MIDAS statistical package (13). The first four sets of data were C_{bd}/C_{fd} and f_d at dialysis equilibrium following treatments *A*, *B*, *C*, and *D*. The fifth set consisted of the pooled C_b/C_f and f for treatments *A–D* corrected back to the corresponding plasma concentration as the blood was withdrawn from the subjects. The pooled data consisted of $N = 419$ samples. Treatments *A*, *B*, *C*, and *D* consisted of $N = 102, 100, 104, 100$, respectively. The discrepancy between the dialysis equilibrium and pooled sample sizes is due to the fact that 13 plasma dialysis samples were either lost or contaminated. However, it was still possible to estimate C_b and C_f values at these time points since the C_t values were known.

RESULTS

Cumulative probability plots corresponding to the free fraction data at dialysis equilibrium for treatments *A*, *B*, *C*, and *D* are shown in Fig. 1. The plots are all curved indicating skewness and nonnormality. Cumulative probability plots corresponding to the bound/free ratio data at dialysis equilibrium are shown in Fig. 2. In all cases the plots are linear over most of the range indicating that the data are normally distributed.

A summary of the percent free fraction and bound/free ratio data corresponding to the C_t values as blood was withdrawn from the subjects is presented in Tables II and III, respectively. Cumulative normal probability plots for the pooled ($N = 419$) C_b/C_f and f data are shown in Fig. 3. Again the bound/free ratios appear normally distributed, while the free fractions appear skewed and nonnormal.

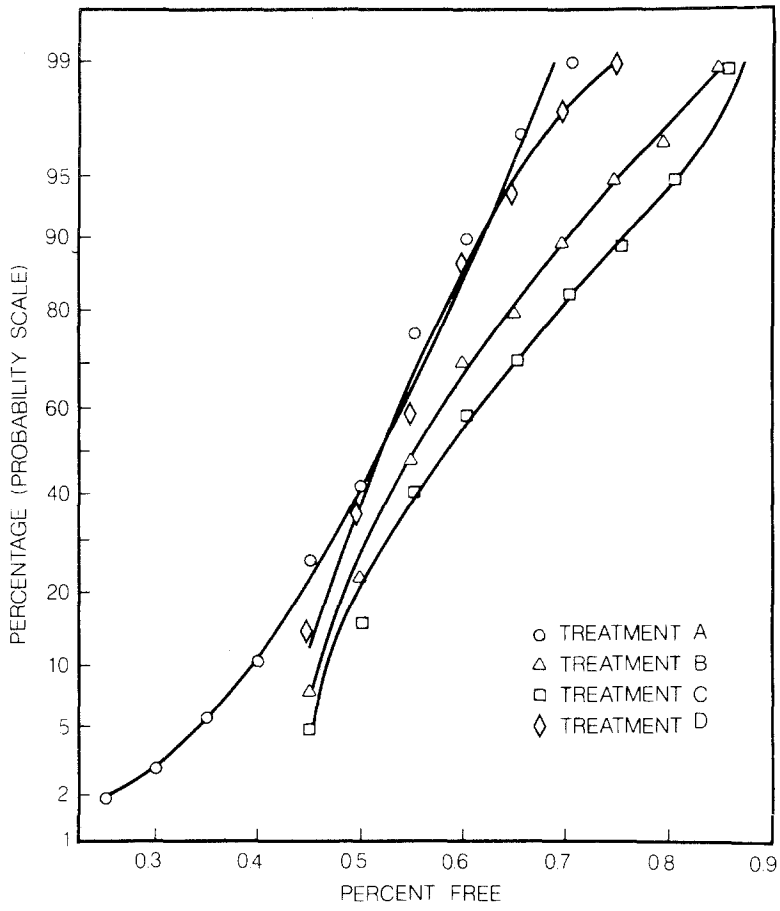

Fig. 1. Plot of cumulative frequency as a percentage of the total on a probability scale vs. percent ibuprofen free at dialysis equilibrium. The curvature indicates that the data are not normally distributed.

The Lilliefors test (14), which is an extension of the Kolmogorov–Smirnov test for normality, was also performed on the above mentioned data. The results of these tests suggest that the bound/free ratio indeed fits the normal distribution assumption more closely than the free fraction in most instances. The distributions of the bound/free ratio at dialysis equilibrium gave the following results. Treatment *A* was significantly different from a normal distribution ($p < 0.05$), but treatments *B*, *C*, and *D* were not significantly different from a normal distribution ($p > 0.5$). The distributions corresponding to the free fraction at dialysis equilibrium for treatments

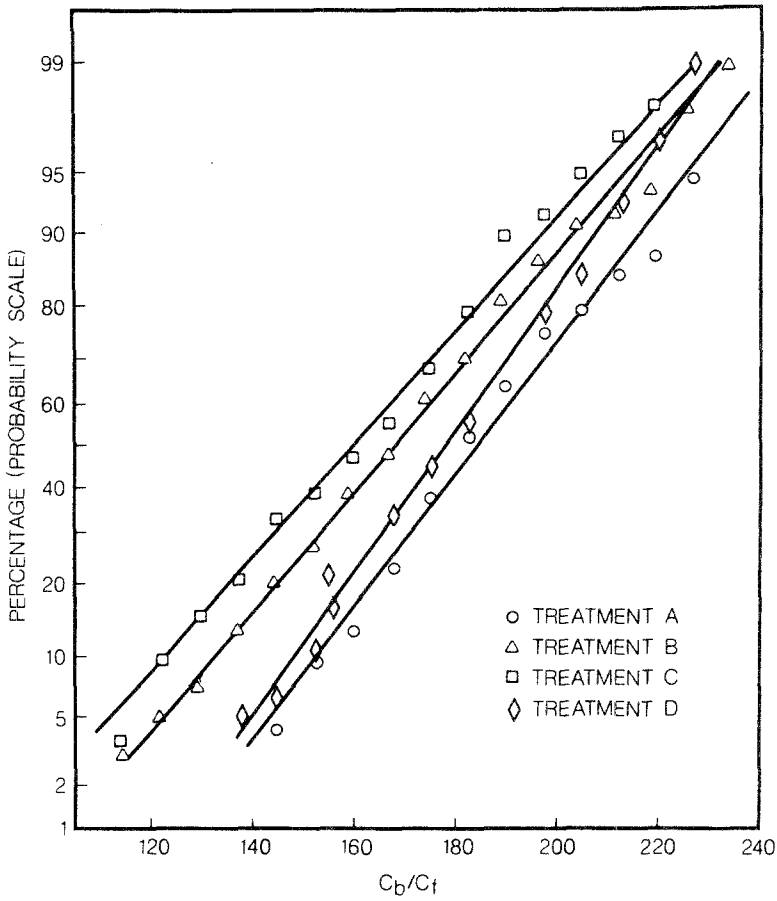

Fig. 2. Plot of cumulative frequency as a percentage of the total on a probability scale vs. bound/free (C_{bd}/C_{fd}) ratio at dialysis equilibrium. Plots indicate that the data are normally distributed.

A and C were highly significantly different from a normal distribution ($p<0.01$), whereas treatments B and D were significantly different at a more conservative test level ($p<0.08$ and $p<0.14$, respectively). When the test procedure was performed on the pooled C_b/C_f and f data, the distribution of bound/free ratios was not significantly different than a normal distribution ($p>0.3$), whereas the free fraction was significantly different ($p<0.01$). Thus, on the basis of two approaches used to investigate normality, the distributions of the bound/free ratio approximate the normal whereas the distributions of the free fractions do not. The authors believe

Table II. Free Fraction Data

	Percent ibuprofen free (100f)			
	Mean	Range	Standard deviation	C.V.%
Solution ($N = 105$)	0.541	0.429–0.643	0.052	9.6
A (1 tablet) ($N = 104$)	0.532	0.433–0.672	0.052	9.8
B (2 tablets) ($N = 105$)	0.566	0.428–0.799	0.072	12.9
C (3 tablets) ($N = 105$)	0.604	0.419–0.933	0.114	18.8
Pooled data ($N = 419$)	0.561	0.419–0.933	0.081	14.6

that the shape of the cumulative distribution plot is much more informative concerning normality or nonnormality than many of the statistical tests in the literature.

By plotting data for individual subjects, as illustrated by Fig. 4 for subject 14, the relationship between bound and free concentrations for a given subject appeared to be independent of the treatment; hence data following treatments *A*, *B*, *C*, and *D* for each subject were pooled (25 to 28 points per subject). Each of these pooled data sets were computer-fitted to Eq. (1). Results are listed in Table IV. The fits of the 15 sets of data to the Scatchard equation with only one class of binding sites were excellent

Table III. Parameters of the Normal Distributions of Bound/Free (C_b/C_f) Ratio

	C_b/C_f ratio			
	Mean	Range	Standard deviation	C.V.%
Solution ($N = 105$)	186[a]	155–233	18.8	10.1
A (1 tablet) ($N = 104$)	189[a,b]	148–230	18.9	10.0
B (2 tablets) ($N = 105$)	178[b]	124–233	22.2	12.5
C (3 tablets) ($N = 105$)	170[b]	106–237	30.0	17.7
Pooled data ($N = 419$)	181	106–237	24.0	13.3

[a]Means do not differ significantly by a *t*-test ($p < 0.2$).
[b]Analysis of variance indicated means differ significantly ($p < .001$).

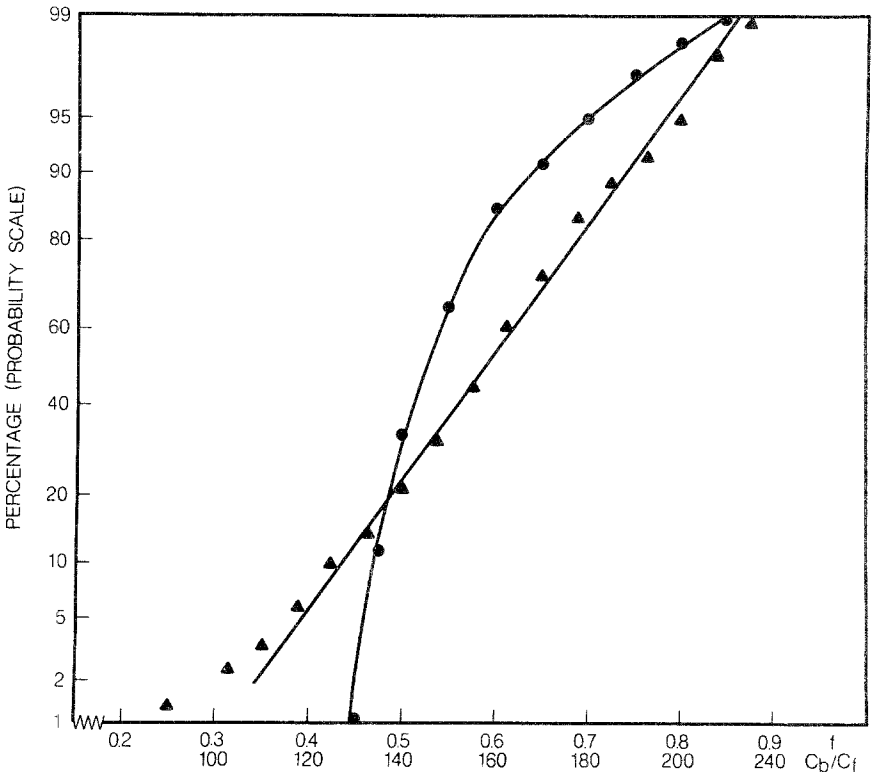

Fig. 3. Plot of cumulative frequency as a percentage of the total on a probability scale vs. percent ibuprofen free for the pooled treatments. Plot of cumulative frequency as a percentage of the total on a probability scale vs. bound/free (C_b/C_f) ratio for the pooled treatments. ●, percent free fraction; ▲, bound/free (C_b/C_f) ratio. Note the curvature indicating skewness in percent free fraction, and the linearity indicating normality in bound/free (C_b/C_f) ratio.

as indicated by the measures of fit and standard deviations of the estimated parameters shown in Table IV. The solid line in Fig. 4 is based on the estimating equation $C_{bd} = 252\ C_{fd}/(1.22 + C_{fd})$, where 252 and 1.22 are the values of $P(1)$ and $P(2)$, respectivey, for subject 14.

DISCUSSION

Vowles and Marchant (15) pointed out that there are important differences in the binding characteristics of serum (or plasma) and human serum albumin with the binding to serum proteins being considerably greater than to two different 4% albumin solutions. They conclude that human serum albumin is not a suitable model for human serum. Although there has been

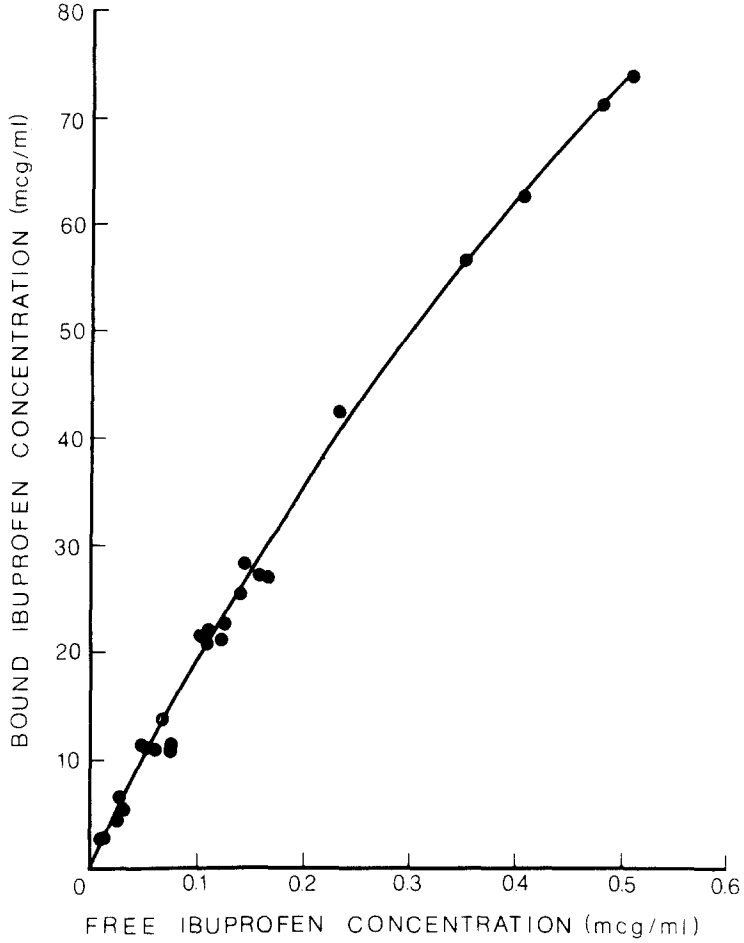

Fig. 4. Plot of bound ibuprofen concentration (μg/ml) vs. free ibuprofen concentration (μg/ml) for subject # 14. Equation of line drawn through the points is $C_{bd} = 252C_{fd}/(1.22 + C_{fd})$ (see Table IV).

considerable literature data on the binding of ibuprofen *in vitro* to human and bovine serum albumin (2–4, 15), there has been little data reported on the binding of the drug *in vivo* to serum or plasma (1, 4, 5, 15).

Our average association constant of 1.76×10^5 M^{-1} (range 1.15×10^5 to 2.76×10^5 M^{-1}) (Table IV) is similar to the k_1 value of 4.73×10^5 M^{-1} reported by Vowles and Marchant (15) but lower than the values of 2.73×10^6 M^{-1} at 37°C reported by Whitlam *et al.* (2) and 1.3×10^6 M^{-1} reported by Kober and Sjöholm (3) for serum albumin. We saw no evidence for a second binding constant as reported by Whitlam *et al.* (2) and Kober

Table IV. Parameter Values and Measures of Fit for Fitting of C_{fd}, C_{bd} Ibuprofen Data to the Scatchard Equation[a]

Subj.	Data points (n)	$P(1)$ (μg/ml)	$P(2)$ (μg/ml)	$P(1)$[b] (μM)	K_a[c] (M^{-1})	Measures of fit: Coeff.[d] of det n	Corr[e]	S[f] (μg/ml)
1	26	175 (13.0)[g]	0.838 (0.091)	848	2.46×10^5	0.988	0.994	2.32
2	28	284 (22.2)	1.49 (0.159)	1377	1.38×10^5	0.992	0.996	2.25
3	26	248 (13.6)	1.37 (0.106)	1202	1.5×10^5	0.997	0.998	1.53
4	27	195 (45.9)	0.995 (0.314)	945	2.1×10^5	0.942	0.970	4.62
5	27	342 (69.4)	1.44 (0.422)	1658	1.43×10^5	0.951	0.975	4.60
6	28	338 (25.5)	1.75 (0.169)	1638	1.18×10^5	0.995	0.997	1.67
7	27	278 (21.9)	1.51 (0.153)	1348	1.36×10^5	0.995	0.997	1.68
8	27	209 (14.7)	0.989 (0.088)	1013	2.0×10^5	0.995	0.997	1.04
9	28	267 (22.0)	1.41 (0.153)	1294	1.46×10^5	0.991	0.995	2.35
10	28	340 (56.0)	1.78 (0.361)	1648	1.15×10^5	0.994	0.996	1.21
11	25	180 (12.3)	0.755 (0.076)	873	2.73×10^5	0.994	0.996	1.93
12	26	208 (20.5)	0.992 (0.126)	1008	2.08×10^5	0.995	0.997	1.09
13	27	191 (9.58)	0.831 (0.060)	931	2.48×10^5	0.994	0.996	1.86
14	27	252 (16.5)	1.22 (0.105)	1222	1.69×10^5	0.996	0.998	1.22
15	27	300 (18.07)	1.51 (0.122)	1454	1.36×10^5	0.994	0.996	2.18
Mean		254	1.26	1231	1.76×10^5			
S.D.		59.3	0.337	287	0.51×10^5			
C.V.(%)		23.3	26.7	23.3	28.9			

[a] $C_{bd}=\dfrac{P(1)C_{fd}}{P(2)+C_{fd}}$, where Both C_{bd} and C_{fd} are in μg/ml. Pooled data from treatments A, B, C, and D employed.

[b] Based on 1 μg/ml = 4.848 μM/L.

[c] Based on $K_a = 10^5/(4.848\times P(2)\ \mu\text{g/ml})$.

[d] Coefficient of detn. $=\dfrac{\sum (C_{bd}-\hat{C}_{bd})^2}{\sum C_{bd}^2-[(\sum C_{bd})^2/n]}$.

[e] Corr = correlation coefficient for the regression of $\hat{C}_{bd}$ on C_{bd}.

[f] $S=\sqrt{\dfrac{\sum(C_{bd}-\hat{C}_{bd})^2}{n-2}}$.

[g] Bracketed numbers are standard deviations of the estimated parameters.

and Sjöholm (3) for serum albumin. Sedman and Wagner (16) discussed the pooling of two or more Michaelis Menten equations such that they would appear to be just one equation. Since Eq. (1) is hyperbolic like the Michaelis Menten equation, the same phenomenon can occur with the Scatchard equation.

It should be noted that with the experimental methods employed, the concentrations C_b and C_f, upon which the binding parameters, % free, and bound/free ratio are based, refer to those existing in plasma as the blood was withdrawn from the subjects. Appropriate corrections were made both for volume changes in the compartments and loss of ibuprofen from the protein compartment during dialysis; hence our data are truly *in vivo* binding data. In the present paper distributional analysis has been performed both with corrected C_b, C_f data and with experimentally obtained C_{bd}, C_{fd} dialysis data. The postdialysis data and the corrected data have similar shaped distributions suggesting that the normality of the bound/free ratios and the skewness of the free fraction are real observations and not an artifact of the mathematical method used to back-calculate concentrations corresponding to the original C_t values.

Although free fraction and percent free or percent bound are used widely as protein binding parameters, we have found that the free fraction of ibuprofen is not normally distributed in five different populations each with 100 to 419 values. However, the reciprocally related (Eq. 3) bound/free ratio was normally distributed for the corresponding five populations. This suggests that tests for significance of difference of population means should more appropriately be carried out using the bound/free ratios rather than the free fractions.

Although we have performed distributional analysis with dialysis equilibrium data in this paper, we would like to stress the importance of expressing bound and free drug concentrations relative to the measured total plasma concentrations. The methods outlined here and elsewhere (8, 10) provide methods to perform these corrections.

APPENDIX 1: DEFINITION OF SYMBOLS

C_t Total drug concentration in plasma before dialysis
C_{td} Total drug concentration in plasma at dialysis equilibrium
C_f Free drug concentration in plasma before dialysis
C_{fd} Free drug concentration in plasma at dialysis equilibrium
C_b Bound concentration in plasma before dialysis
C_{bd} Bound concentration in plasma at dialysis equilibrium which would have been observed if no volume change had occurred

C_p^* Concentration of radiolabeled drug in plasma prior to dialysis (mass/volume)
D_p^* Concentration of labeled drug in plasma prior to dialysis (dpm/ml)
$D_{b'}^*$ Concentration of free labeled drug in the buffer compartment at dialysis equilibrium (dpm/ml)
f Free fraction in plasma before dialysis
f_d Free fraction in plasma at dialysis equilibrium
K_a Association constant of the drug-protein complex if one assumes the drug was bound to only one protein. This is most probably an apparent value since the authors believe that the binding was to more than one entity. Equation (1) adequately describes the binding of ibuprofen in an empirical sense.
$P(1)$ and $P(2)$ parameters (estimated via computer fitting) of Eq. (1)
R V_b/V_p (dimensionless)
SA_{init} Initial specific activity of the drug with units of dpm/mg
V_b Volume of buffer in buffer compartment before dialysis (ml)
V_p Volume of plasma in plasma compartment before dialysis (ml)

APPENDIX 2

The equations used to calculate bound (C_{bd}) and free (C_{fd}) concentrations at dialysis equilibrium are:

$$SA_{init} = \frac{D_p^*}{C_t + C_p^*}$$

$$C_{fd} = \frac{D_{b'}^*}{SA_{init}} = \frac{D_{b'}^*}{D_p^*}(C_t + C_p^*)$$

$$C_{bd} = C_t + C_p^* - C_{fd}(1+R) = \frac{(C_t + C_p^*)(D_p^* - D_{b'}^*(1+R))}{D_p^*}$$

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