Table II—Observed and Predicted Values of Slopes

Retention Time, min	Observed Slope	Predicted Slope
5.2	91.81	90.27
7.8	70.47	68.58
8.0	61.69	59.68

mobile phase, such that changes in retention times occurred with slight changes in methanol concentration. Such changes in retention time would influence the peak heights, thus, peak areas were found to be preferable in the preparation of standard curves.

Peak heights can be used without constantly repeating standard curves, if a correction is made for changes in peak height due to changes in retention time. It is well established that an inverse relationship exists between peak heights and retention time. Different retention times were produced by varying pH, methanol concentration, and I concentration to study this relationship. The flow rate of the mobile phase, however, was always the same. There is a good correlation between the peak heights and the reciprocal of the retention time ($r = 0.9954$), and it is independent of the reason for the change in retention time. Because of the linearity of the response, it should be possible to predict a slope for a new standard curve from a single concentration point. This would preclude the necessity of repeating an entire standard curve when the retention time of sulfinpyrazone changes, although a three-point standard curve would reinforce that reliability. Slopes were predicted from peak heights obtained from a 1-μg/ml drug solution run at different retention times. These values are listed in Table II. Entire standard curves were then run at different retention times and slopes were calculated by linear regression analysis. The latter values are also listed in Table II. The maximum difference between the observed and predicted values of the slope was 2.7%.

It is evident from the results that paired-ion reversed-phase HPLC is a good method for assaying sulfinpyrazone in plasma. Using disposable C_{18} cartridges further simplifies the analysis, since the entire separation of drug from plasma can be accomplished in 2 min. This isolation method also has the advantage of not requiring large volumes of expensive organic solvents as do conventional extraction procedures. While the reported calibration curve used 0.2 μg/ml as the lowest concentration, the sensitivity could easily be increased by increasing the injection volume. This sensitivity is adequate for pharmacokinetic studies or therapeutic drug level monitoring.

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Protein Binding of Caffeine in Young and Elderly Males

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Abstract □ The plasma protein binding of caffeine in young and elderly males was evaluated using an ultrafiltration technique. In spite of a significantly lower plasma albumin concentration in the elderly subjects the observed percent bound (~35%) was essentially identical in both subject groups. The binding of caffeine to human plasma albumin (4.5% w/v) *in vitro* was also examined using ultracentrifugation and it was observed to be bound to the extent of 37.8%. In both the plasma and albumin binding studies the free fraction remained constant over the range of concentrations examined. Although there was no apparent correlation between the percent bound and the albumin concentration in the plasma of either subject group the close agreement between the degree of binding of caffeine to albumin and human plasma indicates that albumin is likely the major plasma binding protein for caffeine.

Keyphrases □ Caffeine—protein binding in young and elderly males, ultrafiltration □ Protein binding—caffeine in young and elderly males, ultrafiltration □ Ultrafiltration—protein binding of caffeine in young and elderly males

It is well known that drug–protein interactions can influence drug pharmacokinetics (1). Since plasma albumin concentration decreases and globulin concentration increases with aging (2, 3), and because a great many drugs are bound reversibly to plasma albumin, the potential importance of age-related changes in protein binding is clear. Of the relatively few studies that have examined the effect of age on protein binding, significant reductions in binding with aging have been reported for meperidine, phenylbutazone, phenytoin, and warfarin, while for phenobarbital, benzylpenicillin, diazepam, desmethyldiazepam, salicylate and sulfadiazine, no alterations in the extent of binding were observed (4). While some studies of caffeine binding *in vitro* (5) and *in vivo* (6, 7) have been reported, no specific examination of possible alterations in its binding characteristics with aging has been made. Since the pharmacological effect of caffeine is probably best related to its unbound fraction (as is true for most drugs), knowledge of any age-related binding differences could prove valuable in helping to interpret the pharmacokinetics of this widely consumed agent, which has received increased attention recently due to its possible role in the treatment of premature apnea (8). The goal of this study was, therefore, to compare the plasma protein binding of caffeine in young and elderly subjects.

EXPERIMENTAL

Subject Selection—Ten healthy, young adult male volunteers ranging in age from 18.8 to 30.0 years and eight healthy, active elderly male volunteers aged 66.0–78.2 years were studied. All subjects were given a physical examination, electrocardiogram, and the following laboratory tests: plasma urea, electrolytes, creatinine, bilirubin, aspartate aminotransferase, alkaline phosphatase, total protein, albumin, creatinine clearance, and complete blood count. In addition, all subjects had a normal health history and were not taking any medication at the time of the study.

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Plasma-Binding Studies—After obtaining informed consent, all subjects were administered a 5-mg/kg dose of caffeine both intravenously[1] and orally[2] on separate occasions ~1 week apart, using a randomized crossover design. The subjects were instructed to abstain from caffeine-containing foods and beverages, tobacco, and alcohol from 72 hr before until 24 hr after each dose of caffeine. Blood samples (10 ml) were collected at 0.25, 1, 4, and 9 hr following the oral dose and at 0.25, 2, 6, and 12 hr following the intravenous dose. Plasma samples were harvested immediately and divided into two aliquots. One aliquot, designated D_t, was stored at −20° until assayed. Three milliliters of the second aliquot was placed in a membrane cone[3], covered[4], and centrifuged at 250×*g* for 30 min, which produced ~1 ml of ultrafiltrate. The ultrafiltrates were tested[5] to ensure that no protein had passed through the cones. If this test indicated that more than a trace of protein was present, another aliquot of plasma was ultrafiltered using a new cone. Previous tests using the Lowry method of assay for protein had indicated that the CF-25 cones removed ~99.8% of the protein from plasma (9). The protein-free ultrafiltrates were then stored at −20°. The ultrafiltrates and D_t samples were assayed in duplicate using a slight modification of a technique described elsewhere (10), the difference being that the protein in plasma samples was precipitated with an equal volume of perchloric acid (12% w/w) in place of the sodium tungstate–sulfuric acid mixture used before, since this precipitant was found to produce a slightly cleaner supernatant (9).

Correction for Membrane Cone Binding—To assess whether significant amounts of caffeine were binding to the membrane cones under the conditions employed in the plasma binding studies, the following experiment was performed. Stock solutions of caffeine at 1, 5, 10, and 20 μg/ml concentrations were prepared in saline phosphate buffer (11). Three-milliliter portions of each stock solution were then placed in membrane cones, which were treated in the usual fashion. The average percent bound over all the cones was then subtracted from each percent bound calculated in the plasma binding experiments to provide a corrected percent bound value.

Albumin Binding Studies—The binding of caffeine to human albumin was studied as follows. A stock solution (5% w/v) of human albumin (essentially fatty acid free)[6] in pH 7.4 saline phosphate buffer was prepared. Sufficient amounts of caffeine in saline phosphate buffer were added to this solution to produce 5 ml of solution containing final concentrations of 1, 5, 10, and 20 μg/ml caffeine in albumin (4.5% w/v). The caffeine–albumin solutions were allowed to equilibrate for 30 min in a 37° water bath. Aliquots (0.5 ml) of these solutions (designated D_t) were then taken and stored at −20°. The remainder of these samples were ultracentrifuged[7] at 4° for 15 hr at 260,000×*g*. Then 0.6 ml of the top layer (designated D_f) was removed, tested[5] to ensure that it was essentially protein free, and stored at −20° until assayed. All samples were assayed in the same manner as the plasma samples.

Calculation of Binding Capacity—The extent of binding was calculated using:

$$\% \text{ Bound } (\beta) = \frac{Db}{Dt} \times 100 = \frac{Dt - Df}{Dt} \times 100 \qquad \text{(Eq. 1)}$$

where Db is the concentration of drug bound to protein, Df is the concentration of free (unbound) drug present in the protein-free ultrafiltrate or upper layer of the ultracentrifuged sample, and Dt is the total concentration of drug (bound plus free) present in the samples prior to ultrafiltration or ultracentrifugation.

Analysis of Data—Differences in the various parameters between the young and elderly groups were assessed using a two-tailed Student's *t* test for unpaired data, with $p < 0.05$ being taken as the minimum level of significance.

RESULTS AND DISCUSSION

The results of the plasma binding studies are summarized in Table I. The values shown have been corrected for the observed degree of binding of caffeine to the membrane cones. It was found that the mean β value for the binding of caffeine to the cones ($n = 18$) was 12.13 ± 0.83% (mean ± *SEM*). The reduction in the total plasma protein concentration observed in the elderly group was not significant ($p > 0.1$), whereas the reduction in the plasma albumin concentration was significant ($p < 0.001$). Both of these observations are consistent with data previously reported (12).

Table I—Comparison of the Plasma Protein Binding of Caffeine in Young and Elderly Males

Parameter	Young Group ($n = 10$) Mean	Young Group ($n = 10$) *SEM*	Elderly Group ($n = 8$) Mean	Elderly Group ($n = 8$) *SEM*	Level of Significance
Age	21.80	1.14	71.20	1.39	$p < 0.001$
Total plasma protein (g/liter)	73.30	1.68	70.00	1.44	NS[a]
Plasma albumin (g/liter)	45.90	0.77	41.13	0.58	$p < 0.001$
Percent bound	35.45	0.73	34.97	0.90	NS[a]
Unbound caffeine concentration (mg/liter)	2.95	0.19	3.06	0.23	NS[a]
Total caffeine concentration (mg/liter)	5.68	0.35	5.78	0.41	NS[a]

[a] Not significant.

In spite of the reduced plasma albumin concentration in the elderly, the observed percentage bound values reported for caffeine did not differ significantly ($p > 0.2$) between the two groups. In addition, the mean percent bound observed here of ~35% correlates well with the value of 31.3% previously reported (6) for 15 healthy male subjects ranging in age from 18 to 71 years. However, both of these values are substantially higher than the value of 15% bound to human plasma previously reported (7).

The following equation (13) illustrates how various factors, including the drug concentration, affect the degree of protein binding:

$$D_t = D_f \left[1 + \frac{nP_t}{\frac{1}{K} + D_f} \right] \qquad \text{(Eq. 2)}$$

where K is the binding association constant (liters per mole), P_t is the total protein concentration, and n is the number of binding sites per protein molecule. When $1/K \gg D_f$ and $n = 1$, Eq. 2 can be simplified to:

$$\alpha = \frac{D_f}{D_t} = \left[\frac{1}{1 + KP_t} \right] \qquad \text{(Eq. 3)}$$

This equation illustrates how the free (unbound) fraction (α) and, therefore, the bound fraction (β) are constants, provided $1/K \gg D_f$. Equation 3 also demonstrates the linear nature of the relationship between the unbound and total drug concentrations. Excellent linear correlations for caffeine binding in the plasma of the young ($r^2 = 0.9393$) and elderly ($r^2 = 0.9088$) subject groups were found (Fig. 1). This figure illustrates the constancy of the degree of binding over the concentration ranged examined, a finding which may also be true for many other drugs over their range of therapeutic concentrations (14). The near-superimposability of the regression lines for the young (slope = 0.5189) and elderly (slope = 0.5337) data is a further demonstration of the similarity in the binding behavior of caffeine in the two groups.

There are at least two plausible explanations for why the observed reduction in plasma albumin in the elderly group did not result in a concomitant reduction in the degree of plasma binding of caffeine. The first possibility is that the amount of drug bound to a given amount of albumin may somehow increase with aging, possibly due to a loss of some endogenous ligand which competes with caffeine for binding sites on albumin. Another possibility is that caffeine may bind to components of plasma other than albumin.

Figure 2 shows a plot of the average percent of caffeine bound to plasma *versus* the measured albumin concentration in each subject and illustrates how there was no apparent correlation between these two parameters. This contrasts with previous findings (6) where a direct linear correlation between the extent of caffeine plasma binding and the serum albumin concentrations was observed in the subject population.

The results of the *in vitro* studies of caffeine binding to plasma albumin are depicted in Fig. 3. These data indicate that caffeine is bound to albumin (4.5% w/v) to the extent of 37.81 ± 1.16% (mean ± *SEM*) with the

[1] Caffeine and Sodium Benzoate Injection, USP, Eli Lilly and Co., Indianapolis, IN 46285.
[2] Caffeine, Baker grade, J. T. Baker Chemical Co., Phillipsburg, NJ 08865.
[3] Centriflo Type CF25, Amicon Corp., Lexington, MA 02173.
[4] Parafilm, American Can Co., Greenwich, CT 06830.
[5] Albustix, Ames Company, Division of Miles Laboratories Ltd., Stoke Poges, Slough SL2 4LY, England.
[6] Product No. A-1887, Sigma Chemical Co., St. Louis, MO 63178.
[7] Model L5-65, Beckman Instruments Inc., Palo Alto, CA 94304.

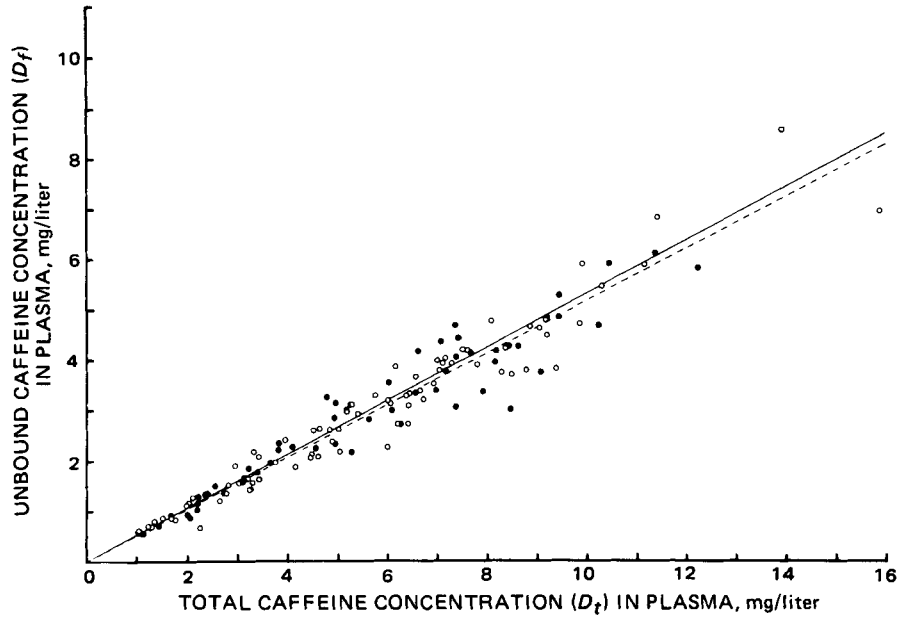

Figure 1—*Relationship between unbound and total caffeine concentrations in plasma of young (○ - - - ○) and elderly (●—●) men.*

percent bound being essentially constant over the concentration range of 1–20 μg/ml, indicating that only a small fraction of the available binding sites is occupied at these concentrations. The close similarity of the mean percent bound values for plasma and albumin implies that albumin is certainly the major plasma binding protein for caffeine, and possibly the only one. The values of the association constant (K) for the respective binding experiments can be calculated by substituting the mean values for β, D_f, and P_t into the rearranged form of Eq. 2 shown below:

$$K = \frac{\beta}{P_t - \beta(P_t + D_f)} \qquad \text{(Eq. 4)}$$

The assumptions involved here are that $n = 1$ and that caffeine only binds to the albumin component of plasma. Under these conditions the value of P_t for plasma in Eq. 4 can be obtained by dividing the average albumin content of plasma by 69,000 (the assumed molecular weight of albumin). The values of K shown in Table II indicate the close agreement of the association constants with one another and with the K value for the

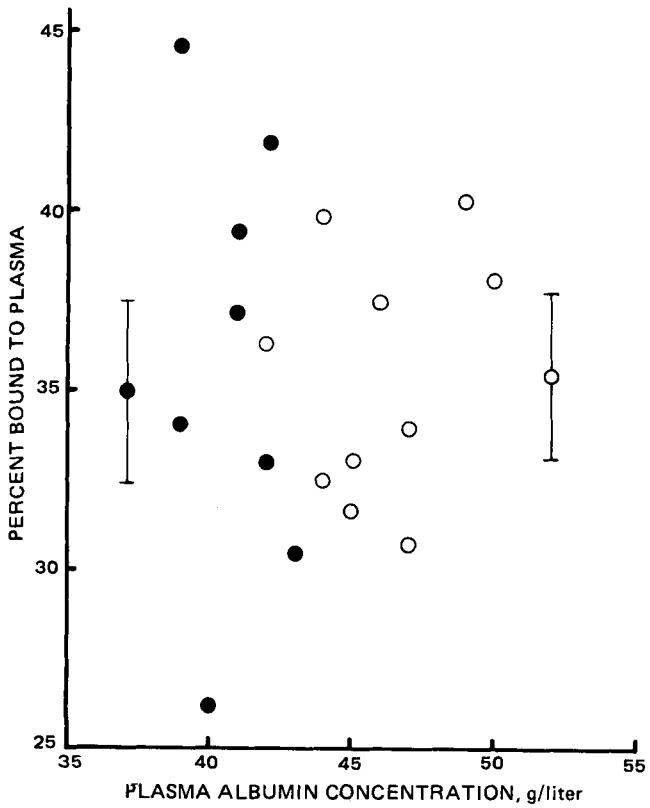

Figure 2—*The effect of plasma albumin concentration on the percentage of caffeine bound to plasma in young (○) and elderly (●) males. Each data point represents the mean of six to eight determinations. The vertical bars represent the means ± 1* SD.

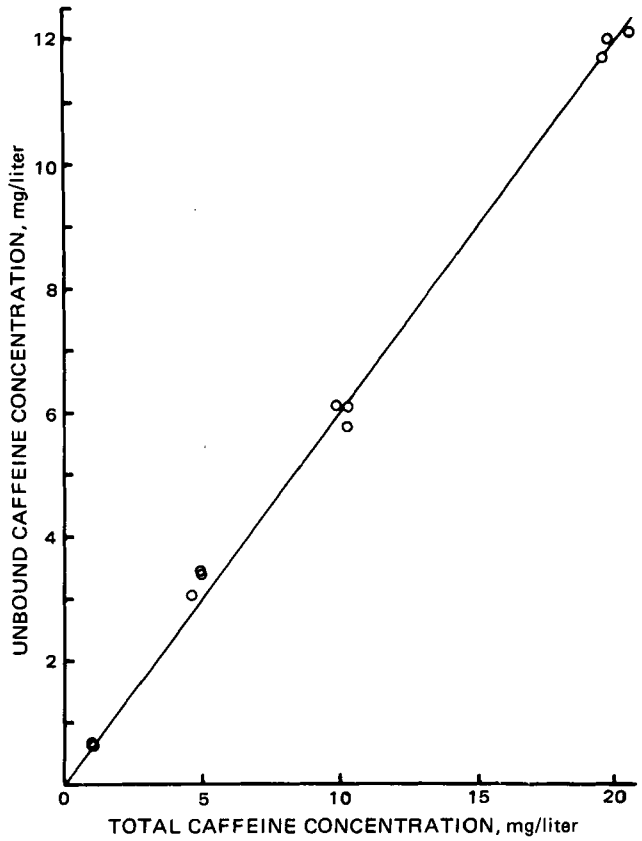

Figure 3—*Relationship between unbound and total concentrations for caffeine binding to human albumin (4.5% w/v)* in vitro. *The regression equation was* $D_f = 0.6012D_t (r^2 = 0.9964)$.

Table II—Calculated Binding Constants for the Interaction of Caffeine with Human Albumin and Human Plasma

Protein-Containing Sample	Binding Association Constant, K (liters/mole)
Human albumin in pH 7.4 buffer	0.958×10^3 (0.932×10^3)[a]
Human plasma from young subjects	0.839×10^3 (0.826×10^3)
Human plasma from elderly subjects	0.918×10^3 (0.902×10^3)

[a] Values in parentheses were calculated using Eq. 3, which assumes that the binding (β) is maximal. The similarity of the respective association constants calculated by each method indicates that drug concentrations are sufficiently low for binding to be nearly maximal.

binding of caffeine to albumin of 1.02×10^3 liters/mole reported previously (16). A 4.5% w/v albumin solution was utilized, since this value represents normal levels in humans and falls within the mean values for plasma albumin found in the two subject groups used in this study. In addition, the plasma binding experiments were performed at 37° using freshly collected (unfrozen) plasma, in view of previous reports (17–19) that the freeze–thaw process may alter drug binding. Thus, the results obtained here should be an accurate indication of the actual binding characteristics of caffeine *in vivo*.

While the binding of caffeine to human plasma is not extensive, and therefore not likely to exert a major influence on its overall pharmacokinetic behavior, a knowledge of the degree of binding over the usual therapeutic range (5–20 μg/ml) could prove useful when monitoring plasma caffeine concentrations in premature infants with apnea (8). These data could also be valuable when studying the effects of various diseases, other drugs, *etc.*, on the clearance of caffeine, since the clearance of unbound drug will be a more accurate indicator of the metabolizing capability of the liver than will the clearance of total (free + bound) drug (20).

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