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DRUG-PLASMA PROTEIN BINDING AND APPARENT VOLUME OF DISTRIBUTION: A COMPLEX RELATIONSHIP

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INTRODUCTION

It is generally believed that only the unbound drug can diffuse out of the vascular compartment and so reach the tissue compartment. Therefore, the magnitude of the apparent volume of distribution (V) of a drug should be directly related to the fraction of unbound drug in plasma (f_p). Based on the physiologic approach to drug distribution developed by Gillette (1971), Wilkinson and Shand (1975) proposed a quantitative relationship between V and f_p,

$$V = V_p + f_p(V_T/f_T) \qquad \text{Eq. (1)}$$

where V_p is plasma volume, V_T tissue volume, and f_T the average fraction of unbound drug in the extravascular space weighted for tissue mass. The value of V_T is equal to the difference between the volume of distribution of unbound drug (Vu) and V_p. When drugs are sufficiently liposoluble, Vu is equal to total body water; and for drugs that do not penetrate cells, Vu is equal to the extracellular space.

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More recently, Wagner (1976), using a pharmacokinetic model, derived a different relationship between V and f_p,

$$V = Vu + f_p Vu(A_{Tb}/Au) \qquad \text{Eq. (2)}$$

where (A_{Tb}/Au) is the ratio of the amount of drug bound to tissue to total unbound drug in the body at any time. However, as demonstrated by Gibaldi and McNamara (1978), this equation is essentially the same as the one proposed by Wilkinson and Shand (1975), based on the work of Gillette (1971).

These relationships have been criticized because plasma proteins are distributed throughout the extracellular space. Therefore, drugs bound to intravascular plasma proteins may indeed be also bound to interstitial proteins. So, it has been claimed that it was important to distinguish between binding to plasma proteins and interstitial proteins. Consequently, Øie and Tozer (1979) have proposed the following relationship:

$$V = V_p(1 + R_{E/P}) + f_p(V_p(V_E/V_p - R_{E/P}) + (V_T/f_T) \qquad \text{Eq. (3)}$$

where $R_{E/P}$ is the ratio of the total number of binding sites or amount of proteins in extracellular fluids outside the plasma to that in the plasma, and V_E is the extracellular space minus the plasma volume (V_p).

Faed (1981), based on similar considerations, proposed the following equation:

$$V = V_p + f_p(V_I/f_I + V_T/f_T) \qquad \text{Eq. (4)}$$

where V_I is the volume of the interstitial fluid, V_T is equal to the total body water minus $(V_P + V_I)$, and f_I is the fraction of the unbound to total amount of drug in the interstitial fluid.

All these relationships show that V is directly related to f_p, but the magnitude of the net change in V, when f_p changes, will depend on many other factors such as extravascular binding and volume, amount of proteins located in the extravascular space, etc. The relative importance of each factor will depend upon the characteristics of each drug. So, theoretically, when V is equal to or smaller than 0.2 l/kg the values of V_p, V_I, f_p, and f_I do have an important role, but when V is larger than 0.2 l/kg the values of V_T and f_T may become much more important. It is beyond the scope of the present work to compare and discuss the advantages of each equation. Equations (3) and (4) are based on a more complex and real physiological

model. However, the parameters included are often impossible to determine. For this reason, throughout the present study we refer to Equation (1).

These relationships are conceptually useful, because they may help to understand the reasons why V changes. The plot of V versus f_p should be linear, with a slope determined by the relative changes of extravascular volumes (V_I, V_E, and V_T) and unbound fractions (f_I and f_T). On the other hand, these equations allow us to extrapolate f_T and so to determine how a certain pathological condition affects f_T. In this way, it was possible to suggest that uremia or nephrosis had apparently no effect on tissue binding of diphenylhydantoin (Gibaldi and McNamara 1978). Indeed, for these calculations it is necessary to assume that effectively there is a linear relationship between V and f_p and that V_p and V_T are not affected by the disease state.

It has been shown that in normal volunteers (Evans, Nies, and Shand 1973), and in patients with liver disease (Branch and Read 1976), the volume of distribution of propranolol is directly related to the unbound fraction. The same direct relationship was observed between warfarin volume of distribution and unbound fraction (Yacobi and Levy 1975). Interestingly, for both drugs the intercept was significantly higher than the theoretical value of plasma volume, suggesting that the changes in f_p were associated to changes in f_T (see Equation (1)), assuming V_T constant.

Unfortunately, the apparent volume of distribution is not always related to f_p. So in man, Klotz, Antonin, and Bieck (1976) could not find any association between the changes in f_p and variations in the apparent volume of distribution of diazepam. In the rat, the distribution of sulfisoxazole (Yacobi and Levy 1979) and dicumarol (Yacobi, Lai, and Levy 1977) does not appear to be associated with their respective plasma unbound fractions.

In a previous study, we investigated in normal volunteers the kinetics of two doses (10 and 40 mg/kg) of sulfamethazine (SMZ) (Souich et al. 1979). When plotting individual values of SMZ volume of distribution versus f_p (Figure 20-1) it is apparent that there is no relationship between these two parameters. In a more recent study (Souich et al. 1983), 12 patients with chronic obstructive pulmonary disease with and without cor pulmonale received 10 mg/kg of SMZ, and again the estimated volume of distribution was not related to f_p values (Figure 20-1). These results prompted us to investigate in conscious normal animals and animals with an hypoxemia and/or hypercapnia or a metabolic acidosis, how these experimental conditions may influence the relationship between the apparent volume of distribution and f_p. The substrates used were SMZ, a weak acid (pKa 7.4) rather hydrosoluble, diphenylhydantoin (DPH), a weak acid (pKa 8.3) but liposoluble, and finally theophylline, a weak base

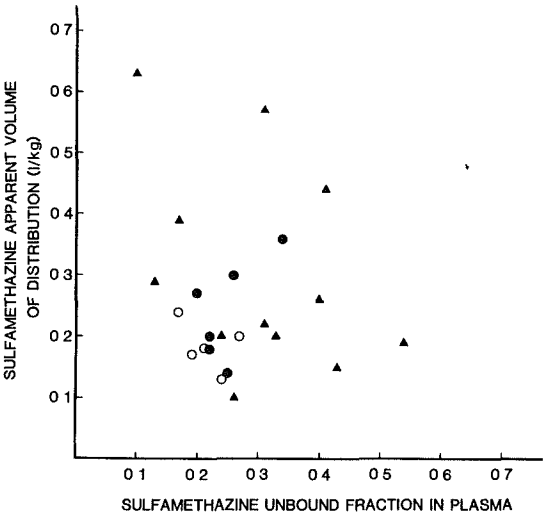

FIGURE 20-1
Sulfamethazine (SMZ) apparent volume of distribution versus SMZ unbound fraction in normal volunteers following a 40 mg/kg (●) or a 10 mg/kg (o) oral dose of SMZ and in patients with chronic obstructive pulmonary disease (COPD) with or without cor pulmonale (CP) (▲). The coefficient of correlation for the normal volunteers was r^2 = -0.4430 and r^2 = -0.4373 following the 40 and 10 mg/kg doses respectively and for patients with COPD r^2 = -0.1466. When all subjects were considered together the coefficient of correlation was r^2 = -0.0233.

(pKa 8.8) hydrosoluble.* The selected doses of these substrates originated apparent first order plasma kinetics.

As shown in Figure 20-2, in control rabbits (breathing air) the values of SMZ apparent volume of distribution did not correlate with changes in SMZ f_p (r^2: -0.0120). The same trend was observed in the groups of rabbits with hypoxemia and/or hypercapnia or metabolic acidosis. Concerning theophylline (T), in control animals as well as in animals with hypoxemia and/or hypercapnia, or with a metabolic acidosis, T appears to be independent of the unbound fraction of the drug, as changes in f_p did not influence T volume of distribution (Figure 20-3). In fact, in rabbits with hypoxemia, T volume of

*Details of the experimental protocols have been published elsewhere—Souich and Courteau (1984); Letarte and Souich (1984).

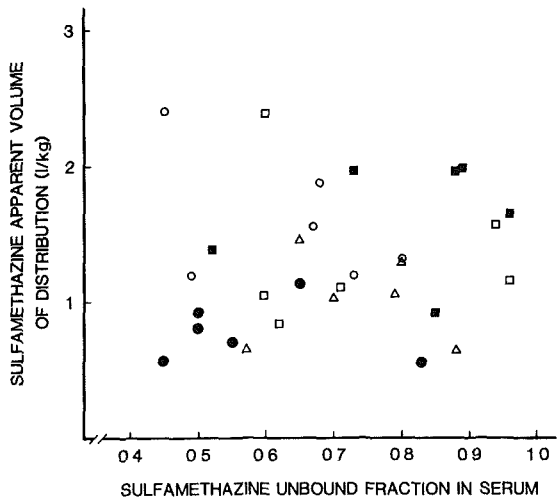

FIGURE 20-2
Sulfamethazine (SMZ) apparent volume of distribution versus SMZ unbound fraction in control rabbits (●), with hypoxemia (□), with hypercapnia (Δ), with hypoxemia combined with hypercapnia (■), and with metabolic acidosis (o). The coefficient of correlation for control animals was $r^2 = -0.0120$ and when all results are considered together $r^2 = 0.0198$.

distribution was increased, despite the fact that the values of f_p were the highest (Letarte and Souich 1984). In control animals, DPH apparent volume of distribution was positively related to DPH f_p ($p < 0.01$) (Figure 20-4). However, this correlation disappeared when the animals were subjected to hypoxemia and/or hypercapnia.*

From the data obtained with these experiments, it is difficult to elucidate why the changes in the apparent volume of distribution are not related to changes in f_p, as many factors may be implicated. With regard to the theophylline study and according to Equation (1), it is possible to predict that the changes in theophylline f_p are accompanied by a similar increase in f_T or by a decrease V_T, thus the apparent volume of distribution of theophylline remains unchanged. Concerning DPH, in the control group (Figure 20-4) the intercept of the plot DPH volume of distribution versus f_p is 0.51 l/kg, and as this value is markedly superior to the real values of the plasma volume (0.076 0.009 l/kg (see Table 20.4). We can conclude that

*Unpublished results.

the changes in f_p are accompanied by proportional changes in f_T or V_T. Despite the fact that the values of DPH f_p were not affected by hypoxemia and/or hypercapnia or metabolic acidosis, the direct relation between DPH f_p and its volume of distribution disappeared.

Indeed, these results raise many questions, such as: what is the explanation for the lack of linear relationship between SMZ volume of distribution and f_p? Why are theophylline f_p changes accompanied by similar changes in f_T or V_T? Why does the relationship between DPH volume of distribution and f_p disappear when the animals are exposed to an atmosphere with a low content of oxygen and/or high content of carbon dioxide, or have a metabolic acidosis? Are the results shown here the consequence of a methodological error?

Among the different factors that may influence the volume of distribution or the f_p of a drug, we should mention the following:

1. In vivo, drug protein binding, as reflected by the f_p, may not be constant. Changes in f_p could be due to a saturation of binding sites secondary to high drug concentrations or hypoalbuminemia, or due to the presence of other substrates competing for the same binding sites, such as metabolites.

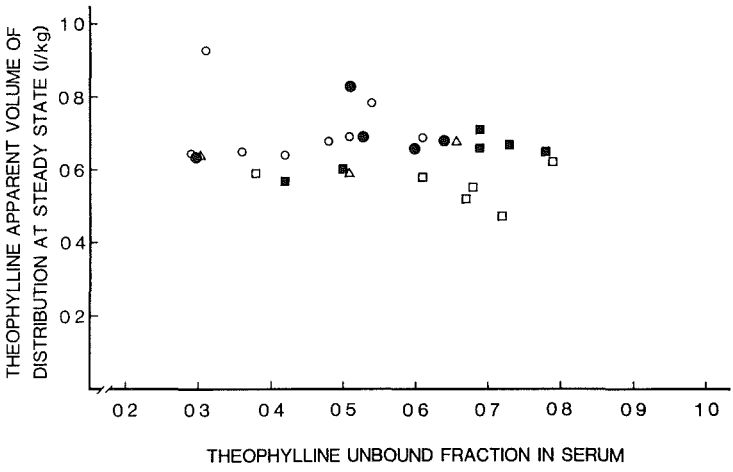

FIGURE 20-3
Theophylline (T) apparent volume of distribution versus T unbound fraction in control rabbits (●), with hypoxemia (□), with hypercapnia (Δ), with hypoxemia combined with hypercapnia (■) and with metabolic acidosis (o). The coefficient of correlation for control animals was $r^2 = 0.0245$ and when all results are considered together $r^2 = -0.0680$.

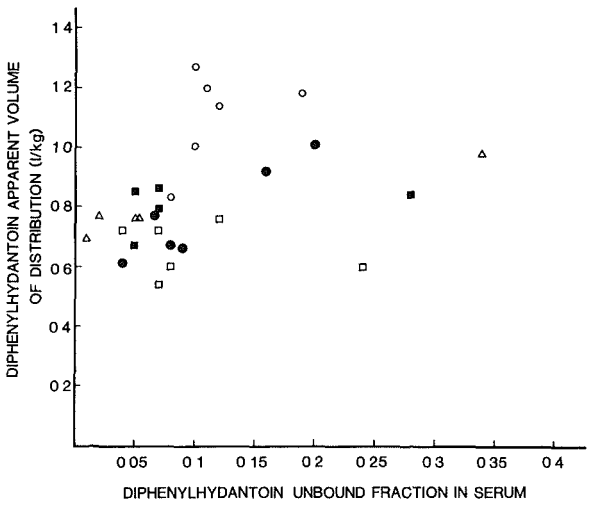

FIGURE 20-4
Diphenylhydantoin (DPH) apparent volume of distribution as function of DPH unbound fraction in control rabbits (●), with hypoxemia (□), with hypercapnia (Δ), with hypoxemia combined with hypercapnia (■), and with metabolic acidosis (o). The coefficient of correlation for control animals was $r^2 = 0.8625$, $p < 0.01$, and when all results are considered together $r^2 = 0.0198$.

2. In vivo, the apparent volume of distribution of a drug may change secondary to changes in the physico-chemical characteristics of the drug—i.e., ionization, or due to changes in tissue perfusion.
3. In vivo, drug effect may influence its apparent volume of distribution or its f_p—i.e., due to hemodynamic or free fatty acid changes.
4. In vivo, the experimental conditions or drug effect may change V_p and/or V_T/f_T.

In analyzing the mechanisms explaining the lack of direct relationship between the volume of distribution of a drug and its f_p, it is important to note that the normal volunteers, as well as patients and the animals used, did not present with hypoalbuminemia.

In normal volunteers, and according to the in vitro studies, following the administration of the 40 mg/kg, or the 10 mg/kg doses of SMZ (Souich et al. 1979), the peak plasma concentrations were high enough to slightly increase f_p (Figures 20-5 and 20-6). This saturation of binding sites may explain why following these doses of SMZ, no relationship was found between SMZ volume of distribution

and f_p (Figure 20-1). The patients with chronic obstructive pulmonary disease also received a 10 mg/kg dose of SMZ, but causes other than saturation of binding sites may also have contributed to abolish the relationship between f_p and SMZ volume of distribution—such as changes in tissue perfusion or even slight changes in SMZ ionization as blood pH was decreased in these subjects (Souich et al. 1983).

When the animals presented a respiratory or a metabolic acidosis, the average value of the plasma pH was 7.26. This pH change will decrease the ratio of ionized to unionized SMZ by 42 percent, and that may have contributed to further change SMZ volume of distribution without a proportional change in f_p.

Our results suggest that the disappearance of the correlation between DPH volume of distribution and f_p in animals with hypoxemia and/or hypercapnia or with metabolic acidosis, must be due to changes in V_p or V_T/f_T because DPH serum protein binding was not

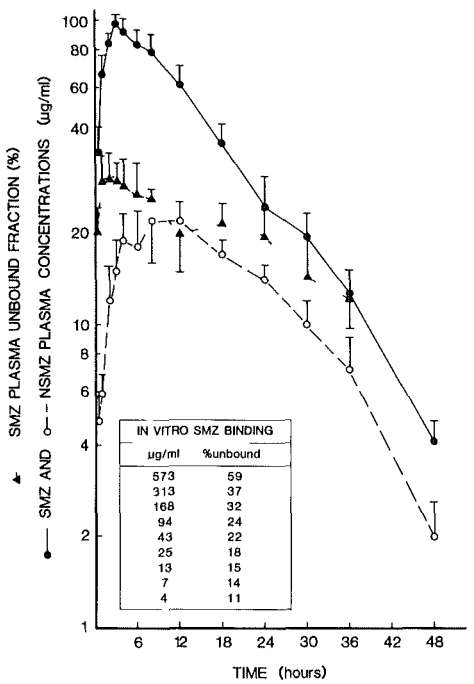

FIGURE 20-5
Average sulfamethazine (SMZ) and N-acetyl SMZ (NSMZ) plasma concentrations and SMZ in vivo unbound fraction as a function of time in normal volunteers following an oral dose of 40 mg/kg of SMZ. In vitro binding studies were carried out in pooled plasma from the same volunteers.

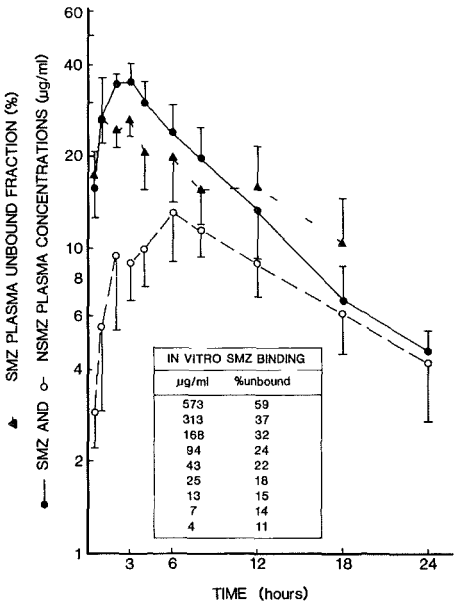

FIGURE 20-6
Average sulfamethazine (SMZ) and N-acetyl-SMZ (NSMZ) plasma concentrations and SMZ in vivo unbound fraction as a function of time in five normal volunteers following an oral dose of 10 mg/kg of SMZ. In vitro binding studies were carried out in pooled plasma from the same volunteers.

altered by these experimental conditions. Under all experimental conditions, where there was an acidosis (pH 7.23) DPH volume of distribution tended to increase or be significantly increased, suggesting that the acidosis influences DPH ionization. This possibility may very well be real as the ratio of ionized to un-ionized DPH decreased from 0.155 to 0.085 when the plasma pH changed from 7.49, under control conditions, to a pH of 7.23. Therefore, following the administration of SMZ or DPH to rabbits, it is possible that the experimental conditions may have changed the volume of distribution by affecting the physico-chemical characteristics of these drugs, besides possible changes in V_p or V_T.

All our in vivo studies were accompanied by parallel in vitro studies. That is, in the experiments where the rabbit was used as a model, a sample of blood was drawn before they received SMZ, theophylline, or DPH and was spiked with amounts of these substrates such as to obtain the same concentrations as observed in vivo. So, the binding of SMZ, theophylline, and DPH was estimated in vitro at

TABLE 20.1
Unbound fraction (mean ± SE) of sulfamethazine (SMZ), theophylline (T), and Diphenylhydantoin (DPH) in serum from man and rabbits. Before (in vitro) and following the administration of 20 mg/kg of SMZ, 2.5 mg/kg of T, or 10 mg/kg of DPH (in vivo). Values represent percent of unbound drug.

	Sulfamethazine		Theophylline	Diphenylhydantoin
	Man	Animals		
In vitro	18 ± 1* 22 ± 1†	28 ± 2	27 ± 3	10 ± 2
In vivo	20 ± 1* 26 ± 2†	58 ± 6‡	51 ± 5**	11 ± 2

Notes:
*SMZ dose of 10 mg/kg.
†SMZ dose of 40 mg/kg.
‡$P < 0.001$, compared to in vitro values (one way analysis of variance).
**$P < 0.01$, compared to in vitro values (one way analysis of variance).

the same concentrations as performed in vivo. In man, a pool of serum of the volunteers was used to carry out in vitro SMZ protein binding studies.* In man, following the 10 mg/kg or 40 mg/kg dose of SMZ, the average f_p estimated in vivo was not different from that observed in vitro (Table 20.1). In the group of control rabbits, the SMZ f_p was increased two-fold when estimated in vivo, compared to the in vitro results ($p < 0.001$). Almost the same difference was observed between theophylline protein binding results obtained in vitro and in vivo (Table 20.1). Interestingly, the DPH f_p values estimated in vitro were identical to the ones estimated in vivo (Table 20.1).

The differences between the binding studies are probably not related to methodology, as in vitro and in vivo samples were always run together, against the same buffer (pH 7.4), ionic strength 0.16, and at 37°C, and incubated for 16 hours when equilibrium dialysis was performed (SMZ), or centrifuged for 15 minutes at 1000 rpm

*Methodological details are described in Souich et al. (1979); Souich and Courteau (1984), and Letarte and Souich (1984).

(theophylline and DPH). On the other hand, in a preliminary study we have controlled the effect of pH (6-8.5) on the binding of SMZ and theophylline and no significant changes were observed. Finally, it is improbable that the observed differences were secondary to hypoalbuminemia as less than 8 ml of blood was drawn between the in vitro sample and the in vivo sample. Therefore, the only difference we can consider is that the in vitro binding studies were done with the parent compound alone, whereas in vivo the parent compound may have generated metabolites, which in turn influenced the f_p. The possibility that the presence of metabolites interferes with the binding of the parent compound is of interest; theoretically that may explain, at least in part, why f_p changes as a function of time. In addition, it may account for individual values of f_p not being associated with the volume of distribution. Indeed, it is important not to forget that under hypoxemia and/or hypercapnia and metabolic acidosis V_p or V_T may have changed and therefore contributed to obscure the relationship between f_p and V.

The studies reported here aimed to determine in the rabbit whether f_p was a constant function of time, and whether the presence of SMZ and theophylline metabolites may change the unbound fraction of these substrates. On the other hand, it was of interest to assess whether hypoxemia and/or hypercapnia might change plasma and interstitial volumes.

MATERIAL AND METHODS

SMZ Protein Binding Studies

Five conscious male New Zealand white rabbits, weighing 2.1 to 3.2 kg, were used to assess in vivo SMZ protein binding, following the administration of an 80 mg/kg intravenous dose of SMZ. Blood was drawn at 3, 6, 9, 20, 40, 75, 115, 150, and 200 minutes and serum was decanted. For the in vitro studies, pooled serum of male New Zealand white rabbits was used. SMZ was spiked in the serum to obtain the following concentrations: 6, 9, 13, 25, 50, 100, 200, 400, and 800 μg/ml and incubated at 37°C for one hour. To investigate the possible interaction between SMZ and N-acetylsulfamethazine (NSMZ) protein binding, we studied the effect of 37.5, 75, and 150 μg/ml of NSMZ on 10, 50, and 100 μg/ml of SMZ.

SMZ protein binding studies (in vitro) with human serum from one subject were done to test whether the acetylated metabolite (NSMZ) could increase SMZ f_p. To test this, several concentrations of SMZ (50, 100, and 150 μg/ml) were incubated for one hour with 25, 50, and 100 μg/ml of NSMZ.

SMZ serum protein binding was determined by equilibrium dialysis, as described elsewhere (Souich et al. 1979). SMZ and NSMZ were determined colorimetrically as described previously (Souich et al. 1979). NSMZ was synthesized from SMZ powder (ICN Pharmaceuticals) as described elsewhere (Souich et al. 1979). All measurements of SMZ protein binding were done in duplicate.

Theophylline Protein Binding Studies

Five conscious male New Zealand white rabbits, weighing 2.3 to 3.3 kg, were used to investigate in vivo theophylline protein binding, following the administration of a 15 mg/kg intravenous dose of theophylline. Blood samples were drawn at 4, 8, 15, 30, 60, 120, 180, 240, and 360 minutes and serum was decanted. For the in vitro studies, pooled serum of male New Zealand white rabbits was used. Theophylline was spiked in the serum to obtain the following concentrations: 5, 10, 15, 20, 80, 200, 500, and 1000 μg/ml and incubated at 37°C for one hour. Theophylline metabolites, 3-methylxanthine (3-MX), 1-methyluric acid (1-MU), and 1,3-dimethyluric acid (1,3-DMU) were purchased from Sigma Chemical Company (Saint Louis, Missouri). To assess the influence of theophylline metabolites on theophylline serum protein binding, several concentrations (6, 12, 18, and 36 μg/ml) of each metabolite alone, or combined, were incubated with 5, 10, 15, and 20 μg/ml of theophylline.

In vitro theophylline protein binding studies with human serum from one subject were done to assess whether its metabolites could increase theophylline f_p. Several concentrations of theophylline (10, 15, and 20 μg/ml) were incubated with 6 and 12 μg/ml of 3-MX, 1-MU, and 1,3-DMU alone or combined.

Theophylline serum protein binding was determined by ultrafiltration, and theophylline in serum was assayed by high pressure liquid chromatography (HPLC) as described elsewhere (Letarte and Souich 1984). All measurements of theophylline protein binding were carried out in duplicate.

Effect of Hypoxemia and/or Hypercapnia on Plasma and Interstitial Volumes

To assess how several experimental conditions may affect plasma and interstitial volumes, four groups of six rabbits were exposed to air, an atmosphere with a low oxygen content, an atmosphere with a high carbon dioxide content, or the combination of the last two conditions. In this way, the control animals had a mean (± SE) partial

pressure of O_2 (PaO_2) and of CO_2 ($PaCO_2$) of 84 ± 2 mmHg and 20 ± 2 mmHg, respectively, and a pH = 7.52 ± 0.01. The group of animals with hypoxemia had a PaO_2 of 51 ± 2 mmHg and $PaCO_2$ of 16 ± 1 mmHg, with a pH = 7.58 ± 0.02. The animals with hypercapnia had a PaO_2 of 90 ± 3 mmHg, a $PaCO_2$ of 62 ± 1 mmHg and a pH of 7.26 ± 0.01. Finally, the group of animals with hypoxemia combined with hypercapnia presented a PaO_2 of 50 ± 2 mmHg, a $PaCO_2$ of 61 ± 2 mmHg and a pH of 7.24 ± 0.01. Following a period of 90 minutes, to equilibrate blood gases, the animals received a 15 mg/kg intravenous dose of inulin. Blood was drawn at 3, 6, 10, 15, 20, 30, 45, and 60 min. Inulin was assayed in plasma by means of a spectrophotometric method—the anthrone method (Davidson and Sackner 1963).

The plasma concentration-time curves for inulin were fitted to a two-compartment open model, with first order distribution and elimination. The volume of the central compartment, corresponding to plasma volume, was estimated by using the following relationship: $V_P = D/A + B$, where D is the dose administered, and A and B are the intercepts of the extrapolation of the elimination and the residual of the distribution phases to the ordinate axis. The volume of the interstitial space was calculated by subtracting V_p from the predicted apparent inulin volume of distribution at steady state (V_{SS}). On the other hand, V_{SS} was estimated by using the equation $V_{SS} = V_P \times (k_{12} + k_{21})/k_{21}$, where k_{12} and k_{21} are the rate constants of intercompartmental distribution. These rate constants were calculated as described by Gibaldi and Perrier (1982).

RESULTS AND DISCUSSION

In the rabbit, following the intravenous administration of 80 mg/kg of SMZ, plasma concentrations ranged between 274 μg/ml and 3 μg/ml at 200 min. Plasma concentrations of NSMZ ranged between 148 μg/ml and 20 μg/ml (Figure 20-7). As shown in the same figure, the fraction of unbound SMZ (f_p) increased from 53 ± 3 percent to reach 69 ± 8 percent and then to decrease to 45 percent. Interestingly, the pattern of changes in SMZ f_p is more closely related to changes in NSMZ plasma concentrations than to the changes of SMZ concentrations. In vitro, SMZ f_p remained rather constant at an average 22 percent, meanwhile SMZ concentrations were lower than 100 μg/ml, although thereafter SMZ f_p increased significantly. The influence of NSMZ on SMZ binding increased as NSMZ and SMZ concentrations were increased, as reflected by an enhancement in SMZ f_p (Figure 20-8). These results confirm previous findings, where following the administration of SMZ (20 mg/kg), the values of

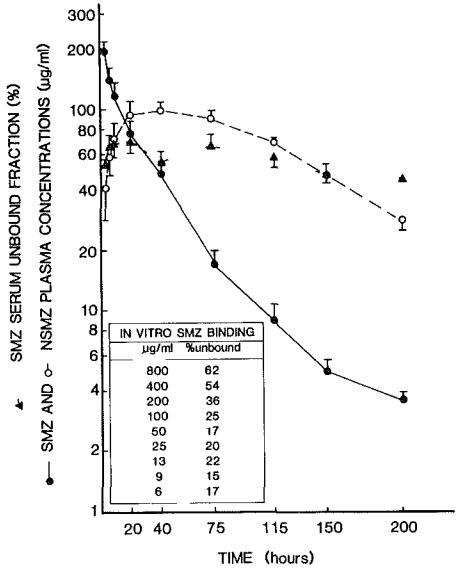

FIGURE 20-7
Average sulfamethazine (SMZ) and N-acetyl SMZ (NSMZ) serum concentrations and SMZ in vivo unbound fraction as a function of time in five New Zealand rabbits following an intravenous dose of 80 mg/kg of SMZ. In vitro studies were carried out in pooled serum for six rabbits.

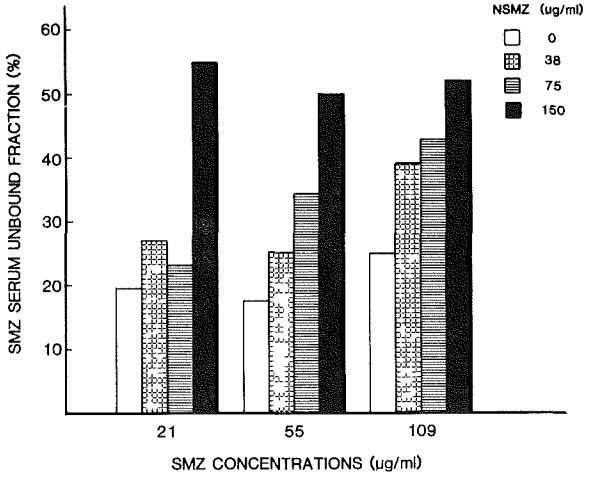

FIGURE 20-8
Effect of N-acetyl sulfamethazine (NSMZ) on sulfamethazine (SMZ) unbound fraction. Increasing concentrations of NSMZ were incubated with several SMZ concentrations (21 ± 3, 55 ± 6, and 109 ± 9 µg/ml; mean ± SE) in pooled serum from five rabbits.

TABLE 20.2
In vitro effect of N-acetylsulfamethazine (NSMZ) on sulfamethazine (SMZ) binding to human serum proteins. Values represent percent of unbound SMZ.

NSMZ (μg/ml)	SMZ		
	50 μg/ml	100 μg/ml	150 μg/ml
0	11	11	13
25	11	10	15
50	12	11	14
100	13	13	17

SMZ f_p estimated in vitro were lower than the ones observed in vivo (Souich and Courteau 1984). These studies in the rabbit suggest that one of the reasons why changes in SMZ volume of distribution are not associated with changes in SMZ f_p is that the metabolite (NSMZ) may be capable of displacing SMZ from its binding sites.

These results, obtained from the animal studies, have some similarities to the ones in man; in vitro the increase in f_p, as SMZ concentrations were enhanced (Figures 20-5 and 20-6), resembles the increment observed in rabbits (Figure 20-7). Furthermore, in man as in rabbits, in vivo SMZ f_p increases with time (Figures 20-5 and 20-6). However, in man the pattern of changes in SMZ f_p function with time appears to be similar to the profile of SMZ plasma concentration. In addition, in man f_p values obtained in vivo do not differ significantly from the ones observed in vitro, as was shown for the rabbits. This suggests that in man NSMZ does not displace SMZ from its binding sites, at least at the concentrations of NSMZ observed in vivo. In vitro studies using human serum confirm that NSMZ does not displace SMZ from its binding sites (Table 20.2). Therefore, in man the changes in f_p appear to be essentially related to concentration-dependent changes. The differences observed between the two species are not surprising if we keep in mind that in the rabbit the ratio of NSMZ AUC to SMZ AUC is 2.55 and in man 0.52 for the 10 mg/kg dose and 0.34 for the 40 mg/kg dose—that is, the rabbit generates a much greater amount of NSMZ than does man. Obviously, the characteristics of the ligand certainly contribute to these differences.

Theophylline (T), when administered intravenously to rabbits at a dose of 15 mg/kg, originated plasma concentrations ranging

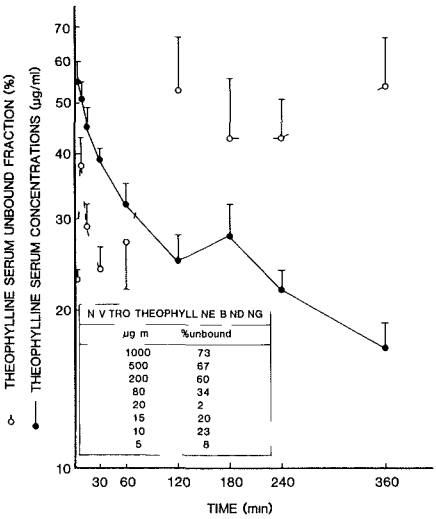

FIGURE 20-9
Average theophylline (T) serum concentrations and T in vivo unbound fraction as a function of time in five New Zealand rabbits following an intravenous dose of 15 mg/kg of T. In vitro studies were carried out in pooled serum of four rabbits.

from 55 ± 5 µg/ml to 17 ± 2 µg/ml at 360 minutes (Figure 20-9). Theophylline f_p was not constant, so an early increase from 24 ± 1 percent to 38 ± 5 percent was observed at 7.5 minutes, and to thereafter decrease to basal values. At 120 minutes, T f_p newly increased to reach values of 53 ± 14 percent. From this point in time, f_p values fluctuated around 48 percent. In vitro, T f_p remained rather constant at a value close to 21 percent until T concentrations reached 20 µg/ml. Thereafter, at 80 µg/ml T f_p increased to 34 percent and reached 73 percent when T concentrations were 1000 µg/ml. These in vitro results may explain the first increase of f_p observed at time 7.5 min, as T concentrations were, on average, 55 µg/ml. That is, at that concentration T binding may already be concentration-dependent. However, we cannot reject the possibility that T having an early sympathicomimetic effect may have raised free fatty acids transiently. Concerning the second increase in T f_p, T serum concentrations appear to be too low to justify this change.

The in vitro studies show that despite the fact that 3-MX does not affect T f_p, 1,3-DMU increases slightly T f_p, an effect which appears to be related to the concentration of this metabolite (Figure 20-10). The effect of 1-MU on T f_p was much more pronounced and was already noticeable at low T and 1-MU concentrations (5 µg/ml

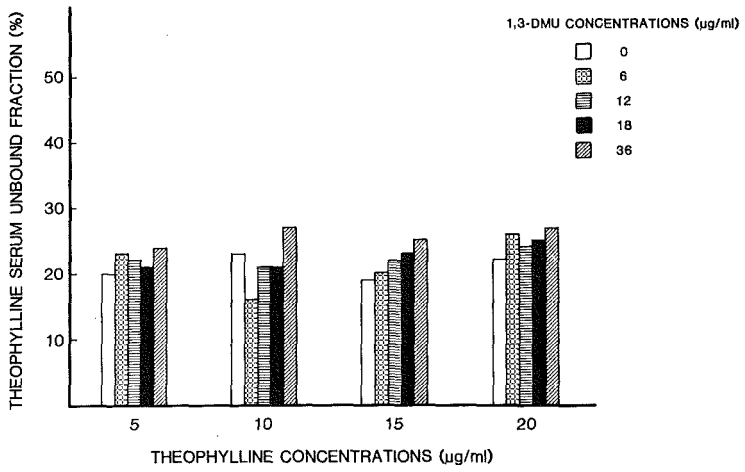

FIGURE 20-10
Effect of 1,3-dimethyluric acid (1,3-DMU) on theophylline (T) unbound fraction. Increasing concentrations of 1,3-DMU (6 to 36 µg/ml) were incubated with T (5 to 20 µg/ml) in pooled serum from five New Zealand rabbits.

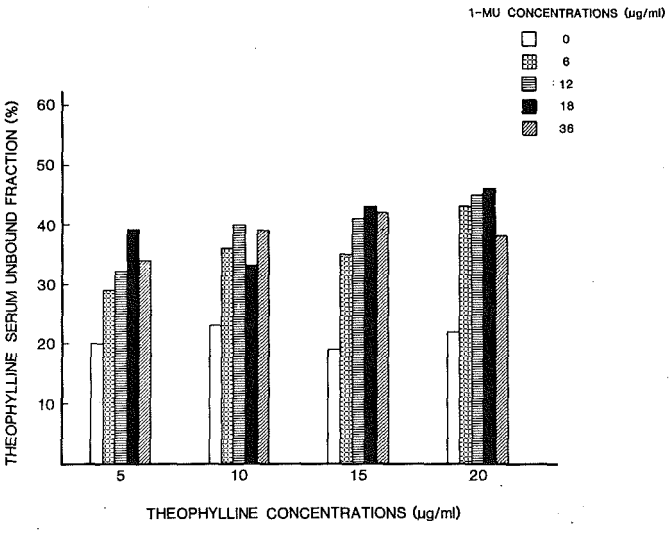

FIGURE 20-11
Effect of 1-methyluric acid (1-MU) on theophylline (T) unbound fraction. Increasing concentrations of 1-MU (6 to 36 µg/ml) were incubated with T (5 to 20 µg/ml) in pooled serum from five New Zealand rabbits.

and 6 μg/ml, respectively). This displacing effect appears to be enhanced when higher (20 μg/ml) concentrations of T are used (Figure 20-11). As T metabolites generated by the rabbit are 1,3-DMU and 1-MU, it was of interest to assess whether the incubation of these two metabolites with T may affect T f_p. As shown in Figure 20-12, the influence of the combination of 1,3-DMU with 1-MU was greater than when any metabolite was considered alone, as f_p was increased by at least a factor of two.

Therefore, it is reasonable to think that the in vivo changes in T f_p may, at least in part, be related to the presence of T metabolites. This hypothesis may explain why T f_p estimated in vivo is greater than when estimated in vitro and furthermore, why in this animal model a single value of T f_p may not be related to T volume of distribution. Indeed, to explain why T f_p changes do not modify T volume of distribution (Figure 20-3) it is necessary to assume that T metabolites affect T tissue binding (f_T) in the same way as they change f_p, so as to keep the ratio f_p/f_T constant (see Equation (1)).

Preliminary results obtained from incubating human serum with increasing concentrations of T (10, 15, and 20 μg/ml) with 3-MX, 1,3-DMU, 1-MU at 6 and 12 μg/ml concentrations alone or in combination, suggest that these metabolites do not modify serum protein binding (Table 20.3).

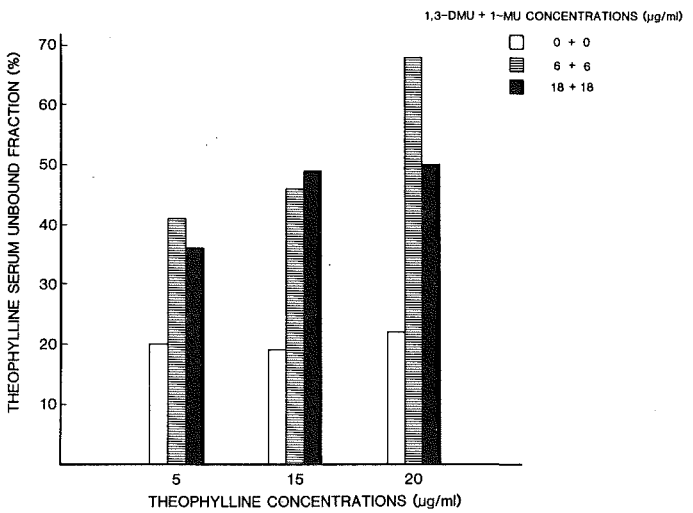

FIGURE 20-12
Effect of 1,3-dimethyluric acid (1,3-DMU) combined with 1-methyluric acid (1-MU) on theophylline (T) unbound fraction. Two concentrations (6 and 18 μg/ml) of 1,3-DMU and 1-MU were incubated with T (5 to 20 μg/ml) in pooled serum from five New Zealand rabbits.

TABLE 20.3
In vitro effect of 3-methylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid (6 and 12 μg/ml) on theophylline binding to human serum proteins. Values represent percent of unbound theophylline.

	Theophylline		
	10 μg/ml	15 μg/ml	20 μg/ml
Without metabolites	22	18	20
3-Methylxanthine (3-MX)			
6 μg/ml	28	26	25
12 μg/ml	21	18	23
1,3-Dimethyluric acid (1,3-DMU)			
6 μg/ml	23	26	26
12 μg/ml	26	20	25
1-Methyluric acid (1-MU)			
6 μg/ml	20	25	25
12 μg/ml	15	23	23
3-MX + 1,3-DMU + 1-MU			
6 μg/ml	15	20	21
12 μg/ml	15	15	15

It is difficult to predict when a metabolite will compete with the parent compound for the binding sites, but in general as the metabolites are more hydrophilic than the parent compound, their bound fraction is usually smaller, so the possibilities for competing with the parent compound will diminish (Jusko and Gretch 1976). In the case where the metabolites are more lipophylic than the parent compound, theoretically the binding of the metabolite may increase and, as a consequence, could displace the parent compound. This has been confirmed with sulfadiazine (Souich et al. 1978), dapsone (Biggs and Levy 1971), and tricyclic antidepressants (Borgå et al. 1969). There are exceptions to this rule, as appears to be the case for theophylline, for disopyramide and its mono-N-dealkylated metabolite (Hinderling, Bres, and Garrett 1974; Aitio 1981), and probably for Tolmetin (Pritchard and Desiraju 1980) and DPH (Letteri et al. 1971) in patients with renal failure. It should be mentioned that the metabolite of a drug may displace a second drug from its binding sites as has been reported with the acetylated metabolite of sulfaphenazole and tolbutamide (Sugita et al. 1984). It might

be still more difficult to predict when these interactions will happen in man. As shown, NSMZ does not appear to affect SMZ binding in vivo or in vitro, and in addition, theophylline metabolites do not influence in vitro theophylline binding.

Hypoxemia and/or hypercapnia did not influence DPH f_p. However, the direct relationship between DPH f_p and DPH volume of distribution disappeared. It was of interest to document whether these experimental conditions might have influenced plasma (V_p) and/or interstitial volumes (VI) and as a consequence V. As shown in Table 20.4, the only significant change was a decrease in VI (35 percent) in hypercapnic rabbits, but as V_p increased slightly, average decrease in extravascular volume (EVV) decreased by only 18 percent. Hypoxemia decreased EVV by 16 percent. However, this difference was not statistically significant. Hypoxemia combined with hypercapnia showed a tendency to increase V_p (19 percent), although as VI decreased slightly, the average EVV increased only by 5 percent. These results do suggest that some experimental conditions may modify V_p and/or VI, and this change may be an additional factor that contributed to obscure the direct relationship between the volume of distribution and f_p. On the other hand, hypoxemia and/or hypercapnia are known factors capable of modifying tissue perfusion (Harper 1965; Broadie et al. 1979; Kendrick, de Haan, and Parke

TABLE 20.4
Effect of hypoxemia and/or hypercapnia on the plasma and interstitial volumes in conscious rabbits

	Plasma Volume (ml)	Percent*	Interstitial Volume (ml)	Percent*
Controls	210 ± 23†	7.6 ± 0.9	309 ± 42	11.1 ± 1.4
Hypoxemia	193 ± 15	6.8 ± 0.7	241 ± 16	8.5 ± 0.7
Hypercapnia	222 ± 26	9.1 ± 1.0	202 ± 43‡	8.1 ± 1.4
Hypoxemia/ Hypercapnia	249 ± 23	10.2 ± 1.0	294 ± 37	11.9 ± 1.3

Notes:

*Percent of body weight.

†Mean ± SE.

‡$P < 0.05$ when compared to control volumes (variance and Dunnett tables for multiple comparisons).

1981; Fink et al. 1977; Roth and Rubin 1976; Vance et al. 1979), suggesting that under these acute experimental conditions the distribution of a drug might be modified. The net effect will depend on the physico-chemical characteristics of the drug, its distribution, and the severity of the hypoxemia and/or hypercapnia. Moreover, the drug itself might change tissue perfusion. All these situations may theoretically change the apparent volume of distribution of a drug without affecting its f_p and therefore the relationship between these two parameters will disappear.

The Figures 20-2, 20-3, and 20-4 show the plot of the values of the volume of distribution function as a single value of f_p estimated in vivo. The validity of such plots is questionable when we take into account that f_p changes with time. To determine whether the lack of association between f_p and the volume of distribution was a consequence of the methodology used, we have tried to correlate the values of the volume of distribution with the average f_p values or with the AUC of f_p. That is, as SMZ and T f_p (Figures 20-7 and 20-8) were estimated in each blood sample drawn, it was possible to calculate the average value of f_p and the AUC of f_p. Neither method ameliorated the correlation between the volume of distribution and f_p. Therefore, we believe that the lack of correlation between the apparent volume of distribution and f_p does not arise from a methodological error such as the misselection of the f_p value.

In conclusion, we have shown that in the rabbit, for SMZ as well as for T, the unbound fraction is not constant. This may, at least in part, be due to a competition between the parent compound and the metabolite. In addition, when using high doses of these drugs it is possible that some degree of saturation of the protein binding sites may have been present and as a consequence, the unbound fraction may increase. On the other hand, we have also shown that under very specific experimental conditions, V_p and VI may change. Finally, it is also possible that under certain experimental conditions (acidosis), the ratio ionized to non-ionized drug may have changed and furthermore, experimental or pathological conditions may change tissue perfusion, and as a consequence the volume of distribution of a drug may be altered. All these factors may have contributed to obscure the relationship between f_p and the volume of distribution. Indeed, these results do show that the relationship between f_p and the apparent volume of distribution is very complex and so, for certain drugs, it may be difficult to understand what determines the changes in the apparent volume distribution by simply using an equation. It is too early to extrapolate these findings to man. However, preliminary results suggest that SMZ or T metabolites do not displace the parent compounds from its binding sites, so the lack of relationship between the volume of distribution and f_p observed in normal volunteers may

be, at least in part, related to the saturation of protein binding sites. The data presented here also show that when studying the characteristics of the protein binding of a drug in vitro results may not always coincide with in vivo observations. Finally, our results also emphasize that in studying protein binding, the appropriate choice of animal model is of utmost importance.

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