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# The Binding Sites on Human Serum Albumin for Some Nonsteroidal Antiinflammatory Drugs[1]

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SUMMARY

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The four antiinflammatory drugs azapropazone, flurbiprofen, ibuprofen, and naproxen all bind very strongly to serum albumin with association constants, $K_a$, of $5.0 \times 10^5$, $5.0 \times 10^6$, $1.3 \times 10^6$, and $1.8 \times 10^6$ $M^{-1}$, respectively. The binding constants were determined with albumin immobilized in microparticles and were shown to be in good agreement with those obtained with equilibrium dialysis. Ibuprofen, flurbiprofen, and naproxen are primarily bound to the diazepam site on the albumin molecule as shown in interaction studies with albumin immobilized in microparticles. This site is shared with, e.g., some antidiabetic agents and benzodiazepines. Azapropazone is primarily bound to the warfarin site, to which also other coumarin derivatives and, e.g., phenytoin and bilirubin are bound. The antiinflammatory drugs studied have small distribution volumes and low free fractions in plasma, which means that displacement from their binding sites may be of pharmacokinetic significance.

## INTRODUCTION

Albumin is the principal drug-binding protein in plasma, binding with high affinity a number of drugs having acidic or other strongly electronegative centra (1). The drugs are bound to at least four different sites of varying specificity (2). The free concentration of a drug in plasma is determined by the association constant, $K_a$, for the binding of the drug to the protein, the number of binding sites, $n$, to which the drug is bound, and the total concentration of the drug and binding protein. The free concentration can also be affected by the presence of other drugs or endogenous compounds, or by diseases, inhibiting the binding to separate sites to varying degrees (3, 4). In order to foresee any inhibition of the binding, it is obviously necessary that the binding sites of the drug on the albumin molecule are known.

The determination of the binding characteristics ($K_a$, $n$) for a drug is often technically complicated, especially for strongly bound drugs. However, we have recently found that proteins can conveniently be immobilized in very small spherical particles of polyacrylamide in such a way that the biological properties are essentially retained. With albumin immobilized in such microspheres (diameter, about 1 μm), a solid phase system is available for simple quantitative determination of association constants (5) and for studies on drug interactions at the protein-binding level. The albumin microparticles have also been used for qualitative characterization of three of the different drug-binding sites on the albumin molecule (6).

In the present work, albumin in microparticles has been used to identify the specific binding sites for some nonsteroidal antiinflammatory drugs and to quantitatively determine their binding characteristics. Some studies have also been made with equilibrium dialysis in order to compare the data obtained with microparticles and to determine the protein binding in serum.

## MATERIALS AND METHODS

*Drugs.* [$^{14}$C]Azapropazone (30 mCi/mmol) was kindly provided by LEO AB, Helsingborg, Sweden; [$^{14}$C]flurbiprofen (4.1 mCi/mmol) and [$^{14}$C]ibuprofen (13.8 mCi/mmol) by Boots Company, Nottingham; and [$^{3}$H]naproxen (18 Ci/mmol) by Syntex Research, Stanford, California. [$^{14}$C]Dicoumarol (16.44 mCi/mmol) was purchased from New England Nuclear, Boston, Massachusetts, and [$^{14}$C]salicylic acid (52 mCi/mmol) from the Radiochemical Centre, Amersham. All labeled drugs had a radiochemical purity >99.5% as controlled by thin-layer chromatography. When necessary the drugs were purified by preparative thin-layer chromatography. Unla-

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beled drugs were placed at our disposal by the respective manufacturers or their representatives in Sweden.

*Serum.* Serum samples were obtained from four healthy, drug-free individuals, one male and three females, 20–25 years of age. The blood was collected through stainless-steel needles directly into glass tubes used for the preparation of the sera, which were pooled and stored frozen until use.

*Human serum albumin (HSA).* Albumin was purchased from AB Kabi, Stockholm. The albumin used in the equilibrium dialysis studies was treated with activated charcoal at pH 3.0.

*Preparation of microparticles with human serum albumin (HSA).* Microparticles of polyacrylamide[2] with immobilized HSA were prepared as described by Ekman and Sjöholm (7). The total concentration of acrylic monomers was 8%, and the fraction of bisacrylamide was 25% in the polymerization mixture.

After polymerization, the microparticles were washed repeatedly with buffer until no protein could be detected by spectrophotometry in the supernatant. The protein content in the particles was determined by amino acid analysis after hydrolysis in 6 M HCl at 105°C for 24 h. In some cases the protein content was estimated from the capacity of the microparticles to bind [$^{14}$C]salicylic acid. Protein leakage was checked by spectrophotometry and generally no leakage could be detected in 3–4 weeks.

*The binding of drugs to HSA in microparticles.* The quantitative binding studies were performed as outlined by Kober *et al.* (5). The drugs studied were dissolved in ethanol and pipetted into centrifuge tubes. The ethanol was evaporated under a stream of nitrogen, and the immobilized albumin suspended in buffer, 0.1 M KCl, 0.005 M sodium phosphate, pH 7.4, was added. The drugs were then redissolved in the "albumin suspension." All drugs studied were shown to be completely redissolved in this way within 1 h without any addition of ethanol. In all binding studies the albumin concentration was 1 mg/ml (15 $\mu$M) except with flurbiprofen, where the concentration was 2 mg/ml (30 $\mu$M) in order to improve the measuring conditions.

The qualitative interaction studies were, in principle, performed as described earlier (6). The displacing drug was dissolved in ethanol and added to the test tubes at the same time as the radioactively labeled marker, with subsequent evaporation under nitrogen as described above. The concentration of HSA was 1 mg/ml (15 $\mu$M) except in experiments with [$^{14}$C]dicoumarol, where the albumin concentration was 2 mg/ml (30 $\mu$M). The molar ratio between the marker (i.e., the radioactively labeled drug) and HSA was 0.5:1 (0.2:1 in the naproxen study), and the displacing drug was added to give drug:HSA molar ratios of 0.2:1 (only in the naproxen study), 0.5:1, 1:1, 2:1, and 5:1. After centrifugation the radioactivity in the supernatant was determined by liquid scintillation counting.

Unspecific binding to polyacrylamide was tested with microparticles not containing HSA. No drug used in this study was found to bind unspecifically to such microparticles.

[2] U.S. patent 4,061,466; British patent 1,533,579.

Due to the chemical instability and light sensitivity of azapropazone, all samples containing this drug were protected from light as far as possible.

*Equilibrium dialysis.* The protein binding in human serum as well as the binding to isolated charcoal-treated HSA were determined by equilibrium dialysis at room temperature, as described earlier (3). The drugs were dissolved in the protein solution by the same procedure as in the study with microparticles described above. The time used for equilibration was 6 h for ibuprofen and flurbiprofen and 12 h for naproxen. The binding was related to the albumin concentration, which was determined after the dialysis by immunochemical quantitation according to Mancini *et al.* (8) using M-Partigen plates obtained from Hoechst Behring Werke AG.

None of the drugs was found to bind to the membrane or to the walls of the dialysis cells.

*Evaluation of the binding data.* The binding data obtained with the different drugs were analyzed according to Scatchard (9). The equation

$$r/(D) = n \cdot K_a - r \cdot K_a$$

was used, where $r$ is the number of moles of bound drug per mole of albumin, $n$ the number of binding sites, $K_a$ the association constant for the drug–albumin complex, and $(D)$ the concentration of free drug.

In some interaction studies the Rosenthal plot (10) was used based on the following equation:

$$(DP)/(D) = n \cdot M \cdot K_a - (DP) \cdot K_a,$$

where $(D)$, $K_a$, and $n$ are as defined above, $(DP)$ is the concentration of bound drug, and $M$ denotes the concentration of macromolecules. This plot, which is a modified form of the Scatchard plot, was used when essentially only the $K_a$ value was wanted and the exact protein concentration was not determined.

In both plots, $K_a$ was calculated from the slope of the straight lines formed by the experimental points close to the $Y$ axis. Thereby, the influence from secondary binding was minimized. Such secondary binding generally was very low and unsignificant, and only with ibuprofen was a small curvature seen at values with $r < 0.6$.

## RESULTS

*Determination of binding characteristics.* The binding characteristics of the four drugs, as obtained from Scatchard plots, are summarized in Table 1. The results from the equilibrium dialysis studies are in good agreement with those obtained with microparticles. Examples of Scatchard plots for the binding of azapropazone, flurbiprofen, ibuprofen, and naproxen to HSA immobilized in microparticles (1 mg/ml or 15 $\mu$M) are shown in Figs. 1 and 2. Flurbiprofen is bound to HSA at one primary binding site with an association constant, $K_a$, of $5.0 \times 10^6$ M$^{-1}$. Naproxen is also very strongly bound to albumin at one primary binding site ($K_a = 1.8 \times 10^6$ M$^{-1}$).

Included in Fig. 1 is the binding of flurbiprofen to charcoal-treated HSA as determined by equilibrium dialysis. Both ibuprofen and azapropazone show a high affinity to HSA, $K_a$ being $1.3 \times 10^6$ and $5.0 \times 10^5$ M$^{-1}$, respectively, and as can be seen in Fig. 2, they both have

TABLE 1

*Binding characteristics for some antiinflammatory drugs*

The data have been obtained from Scatchard plots. In serum the binding has been related to the albumin concentration.

| Sample | Method | Flurbiprofen | | | Ibuprofen | | | Naproxen | | | Azapropazone | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $n$ | $K_a$ | $n \times K_a$ | $n$ | $K_a$ | $n \times K_a$ | $n$ | $K_a$ | $n \times K_a$ | $n$ | $K_a$ | $n \times K_a$ |
| | | | $M^{-1} \times 10^{-6}$ | $M^{-1} \times 10^{-6}$ | | $M^{-1} \times 10^{-6}$ | $M^{-1} \times 10^{-6}$ | | $M^{-1} \times 10^{-6}$ | $M^{-1} \times 10^{-6}$ | | $M^{-1} \times 10^{-6}$ | $M^{-1} \times 10^{-6}$ |
| HSA | Microparticles | 1.0 | 5.0 | 5.0 | 1.0 | 1.3 | 1.3 | 1.0 | 1.8 | 1.8 | 1.0 | 0.5 | 0.5 |
| Charcoal-treated HSA | Equilibrium dialysis | 1.3 | 4.1 | 5.4 | 1.1 | 1.2 | 1.3 | 1.4 | 1.4 | 1.9 | | | |
| Human serum | Equilibrium dialysis | 1.4 | 4.1 | 5.7 | 1.0 | 1.0 | 1.0 | | | | | | |

one primary binding site on the albumin molecule. Also shown in Fig. 2 is the binding of ibuprofen to serum as determined by equilibrium dialysis. In serum, the number of primary binding sites as calculated from the HSA concentration is the same but the association constant is somewhat lower than with isolated albumin.

Due to the instability and light sensitivity of azapropazone, $K_a$ determinations using equilibrium dialysis were not performed. The $K_a$ for the binding of naproxen to human serum was not determined because of the difficulties in obtaining the $^3H$-labeled isotope sufficiently pure. The high binding constant for the albumin–naproxen complex ($K_a = 1.8 \times 10^6$ $M^{-1}$) gives a binding in undiluted serum of about 99.5% in the low concentration region used for the determination of the primary binding constant. Thus, very small amounts of radioactively labeled impurities will significantly influence the results (11, 12). With microparticles and charcoal-treated HSA, lower protein concentrations were used, resulting in lower binding degrees of the labeled naproxen and less significance of the impurities.

*Drug interaction studies.* The displacement of the four antiinflammatory drugs from HSA in microparticles was studied by determining the $K_a$ value for the primary binding site in the presence of a displacing drug. The displacer was added to give a molar ratio of displacer: HSA of 0.5:1 to assure that the displacer would be bound mainly to its primary site on HSA. As an example the $K_a$ determinations in the form of Rosenthal plots for azapropazone alone, and in the presence of some other drugs, are shown in Fig. 3. The binding was unchanged on the addition of diazepam, flurbiprofen, ibuprofen, and naproxen, while the addition of warfarin gave a decreased $K_a$ value. The intercept on the $X$ axis in the presence of warfarin was the same as for azapropazone alone, indicating that the number of binding sites was unchanged.

The results obtained in separate experiments with azapropazone, flurbiprofen, ibuprofen, and naproxen are summarized in Table 2. They show that flurbiprofen, ibuprofen, and naproxen are bound to the same primary site on albumin and, furthermore, that this site is the same as for diazepam. The primary binding site for azapropazone is the same as that for warfarin and is not influenced by the presence of the other three antiinflammatory drugs.

A more qualitative approach to the study of drug interactions is shown in Figs. 4 and 5, better simulating the practical situation. In Fig. 4 the interaction on the protein binding level between azapropazone and the anticoagulant drug dicoumarol is shown. Dicoumarol is used as marker and azapropazone is added as the displacing drug. The molar ratio of marker:HSA was 0.5:1,

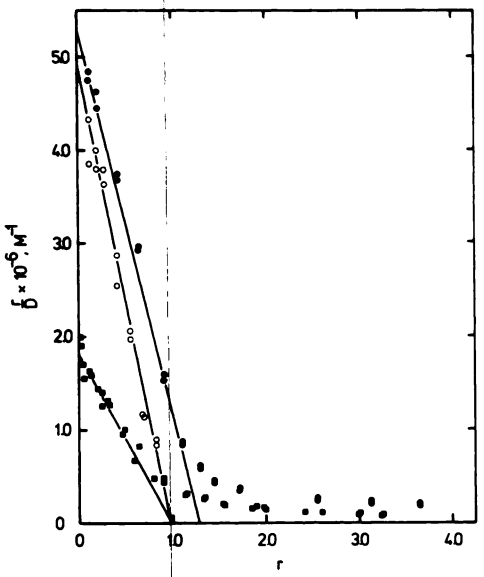


FIG. 1. *Scatchard plots for the binding of flurbiprofen* (○——○) *and naproxen* (■——■) *to HSA immobilized in microparticles and for the binding of flurbiprofen to charcoal-treated HSA as determined by equilibrium dialysis* (●——●)

All determinations were made in a 0.005 M sodium phosphate buffer, pH 7.4, containing 0.1 M KCl. The temperature was 20–22°C.

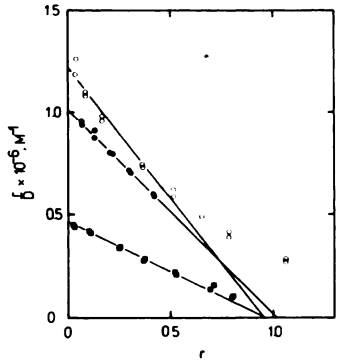


FIG. 2. *Scatchard plots for the binding of azapropazone* (■——■) *and ibuprofen* (○——○) *to HSA immobilized in microparticles and for the binding of ibuprofen in serum as determined by equilibrium dialysis* (●——●)

The experimental conditions were the same as in Fig. 1.

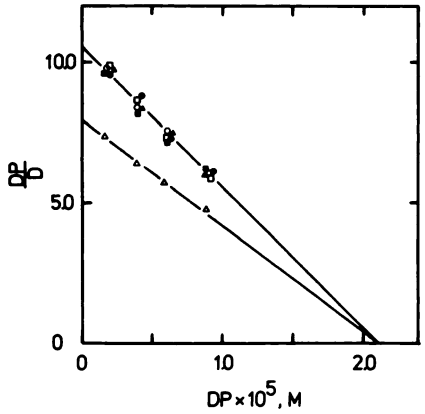


FIG. 3. *Rosenthal plots showing the binding of azapropazone to HSA immobilized in microparticles alone (●—●) and in the presence of diazepam (■—■), flurbiprofen (□—□), ibuprofen (○—○), naproxen (▲—▲), and warfarin (△—△)*

The study was carried out at 20–22°C in a 0.005 M sodium phosphate buffer containing 0.1 M KCl, pH 7.4. The albumin concentration was estimated to be 1 mg/ml (15 μM) and the displacing drugs were added to give drug:HSA molar ratios of 0.5:1.

and the displacer was added to give molar ratios of displacer:HSA of 0.5:1, 1:1, 2:1, and 5:1. The drugs compete for the same site and the results show that azapropazone is effectively displacing the marker, in spite of the lower affinity ($K_a$ of dicoumarol is $3 \times 10^6$ M$^{-1}$; Ref. 13).

In Fig. 5 the displacement of naproxen is shown. The molar ratio of naproxen:HSA was 0.2:1 to assure that naproxen would be bound mainly to its primary site on albumin. The displacing drugs were then added to give drug:HSA molar ratios of 0.2:1, 0.5:1, 1:1, 2:1, and 5:1. As can be seen, the free fraction of naproxen is increased on the addition of flurbiprofen, ibuprofen, or diazepam, while no displacement of naproxen can be observed when azapropazone or warfarin is added.

*Computer calculations.* Some theoretical calculations of drug displacement reactions were made with a computer and displayed as the variation of the relative free fraction of the marker with increasing total concentrations of displacing drug. The calculations were based on the expressions describing the association constants, $K_a$, which were combined. Two different interaction situations are exemplified in Fig. 6. As marker, a drug having

TABLE 2

*Displacement of antiinflammatory drugs*

Displacement from the primary binding site as determined by Rosenthal plots in the presence of a displacer. The HSA concentration was 1 mg/ml (15 μM) (with flurbiprofen, 2 mg/ml or 30 μM), and the molar ratio of displacing drug:HSA was 0.5:1.

| Markers | Displacing drugs | | | | | |
|---|---|---|---|---|---|---|
| | Warfarin | Azapropazone | Diazepam | Flurbiprofen | Ibuprofen | Naproxen |
| Azapropazone | +[a] | | 0 | 0 | 0 | 0 |
| Flurbiprofen | 0 | 0 | + | | + | + |
| Ibuprofen | 0 | 0 | + | + | | + |
| Naproxen | 0 | 0 | + | + | + | |

[a] (+) Displacement reaction causing a decrease in the $K_a$ for the marker; (0) no displacement.

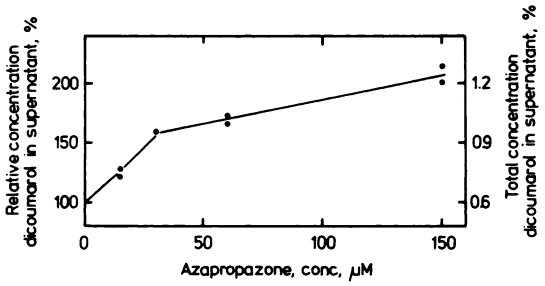


FIG. 4. *Displacement of dicoumarol (15 μM) from HSA in microparticles (2 mg/ml or 30 μM) by azapropazone which was added at 0.5:1, 1:1, 2:1, and 5:1 molar ratios*

a $K_a$ value of $2 \times 10^6$ M$^{-1}$ was used. As displacers, two drugs with $K_a$ values of $5 \times 10^6$ and $2 \times 10^5$ M$^{-1}$ were chosen to illustrate the influence of the displacers' $K_a$ values. The protein concentration used in the calculation was 1 mg/ml (15 μM) and the marker was present at a 0.2:1 molar ratio to albumin. In the first case both the displaced drug, i.e., the marker, and the displacing drugs are assumed to have only one and the same binding site on albumin. As can be seen in Fig. 6, the curve obtained with the displacing drug having a $K_a$ value of $5 \times 10^6$ M$^{-1}$ has a sigmoidal shape with a much steeper slope than the curve for the displacer with $K_a = 2 \times 10^5$ M$^{-1}$.

In the second case the marker drug is assumed to have a second binding site with $K_a = 3.5 \times 10^5$ M$^{-1}$ in addition to the primary one with $K_a = 2 \times 10^6$ M$^{-1}$. The displacing drugs are assumed to interact only with the primary binding site of the marker. The consequence of the second binding site of the marker is a decrease in the displacement, but the shapes of the curves obtained closely resemble those obtained in the first case with only one binding site for the marker.

## DISCUSSION

The four nonsteroidal antiinflammatory drugs azapropazone, flurbiprofen, ibuprofen, and naproxen are all extensively bound to serum proteins with binding degrees above 99%. Serum albumin is considered to be the main

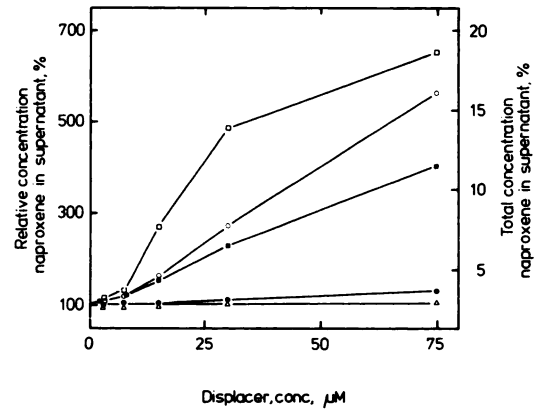


FIG. 5. *Displacement of naproxen (3.0 μM) from HSA in microparticles (1 mg/ml or 15 μM) by azapropazone (●—●), diazepam (■—■), flurbiprofen (□—□), ibuprofen (○—○), and warfarin (△—△)*

The displacing drugs were added to give drug:HSA molar ratios of 0.2:1, 0.5:1, 1:1, 2:1, and 5:1.

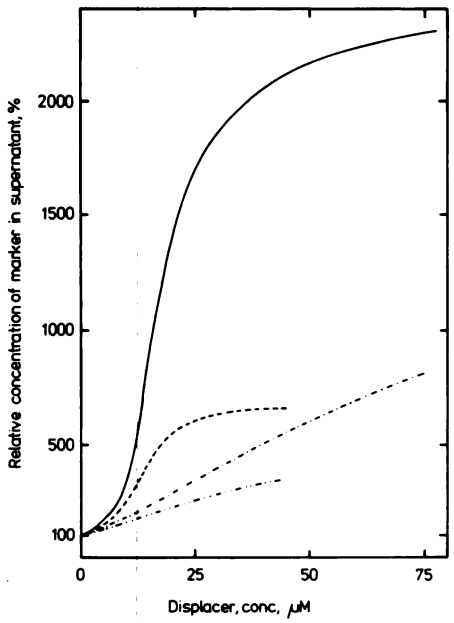


FIG. 6. *The computer-calculated displacement of a marker (3.0 μM) from HSA (1 mg/ml or 15 μM) under different conditions*

(A) The marker has one binding site with $K_a = 2 \times 10^6$ M$^{-1}$ and the displacer is bound to the same site with $K_a = 5 \times 10^6$ M$^{-1}$ (——) or $2 \times 10^5$ M$^{-1}$ (–·–·–). (B) The marker has an additional binding site with $K_a = 3.5 \times 10^5$ M$^{-1}$. The interactions with the displacing drugs with $K_a = 5 \times 10^6$ M$^{-1}$ (– – –) or $2 \times 10^5$ M$^{-1}$ (–·–·–·–) involve only the primary site of the marker.

binding protein in all cases (14–17). This means that these drugs may be potentially involved in drug interactions on the protein binding level which may be of pharmacokinetic significance (1, 18). Such drug interactions will, however, only occur when the drugs bind to the same primary binding site. The quantitative effects of the displacement will also depend on the relative magnitude of the association constants of the interacting drugs. The clinical significance of such interactions will also depend on other factors such as the elimination mechanisms (19) and the tissue binding (20). Interactions involving secondary sites may occur *in vivo*, as well as allosteric effects ("energetic coupling") between different sites on the same molecule. For most drugs secondary sites will not qualitatively affect the displacement within therapeutically relevant concentration ranges, but will have an attenuating effect as shown in Fig. 6. Such a buffering effect will depend on the relative magnitude of the $K_a$s. When allosteric phenomena are effective, the association constants are changed for the protein molecules, which are allosterically modified, but the changes are generally limited (21) and the effects will probably not significantly change the pharmacokinetics.

In the present work the association constants, $K_a$, for the binding of these antiinflammatory drugs to serum albumin, and in some cases to serum, have been determined. Considering the large experimental problems involved in the determination of very high binding constants (see Table 1), the $K_a$ values found for ibuprofen and naproxen closely correspond to those reported earlier (20, 25). However, the value for ibuprofen is considerably higher than reported by Mills *et al.* (14). Also, for flurbiprofen the value is much higher than that reported earlier by Risdall *et al.* (16).

In the evaluation of possible binding interactions between drugs, it is self-evidently essential to know the binding proteins and the identity of the binding sites. Sudlow *et al.* (23) have identified two sites, designated site I and site II, on the HSA molecule. In a previous study (6), we have characterized three sites on HSA, the diazepam, digitoxin, and warfarin sites, named after the markers used. In addition, indications were presented that there exists a fourth site, to which tamoxifen primarily is bound. That study showed that flurbiprofen, ibuprofen, and naproxen effectively displaced diazepam and that azapropazone and naproxen displaced warfarin. At higher drug concentrations where the drug:HSA molar ratios exceeded 1:1, flurbiprofen and ibuprofen also displaced warfarin.

In the present work, we have shown, as summarized in Table 2, that flurbiprofen, ibuprofen, and naproxen all bind to the same primary binding site, i.e., the diazepam site (site II), which is in accordance with the results reported by Sudlow *et al.* (23). Azapropazone is bound to the warfarin site (site I). The effects seen on the warfarin binding from the other drugs can be ascribed to interaction at their secondary binding sites.

Several reports have been published of protein binding interactions between the nonsteroidal antiinflammatory drugs and the anticoagulant drug warfarin (24–28). Azapropazone, ibuprofen, and naproxen have been shown *in vitro* to increase the free fraction of warfarin in serum and also to cause displacement of warfarin from serum albumin. *In vivo*, ibuprofen and naproxen have only a minor effect on the binding of warfarin, and no significant clinical effect on the anticoagulant action has been seen (26–28). In fact, such interactions are hardly to be expected, considering that the drugs bind to separate sites on HSA. On the other hand, azapropazone is more likely to cause serious interactions at the binding level with the highly bound coumarine derivatives. Significant interactions with warfarin have also been detected after azapropazone administration (29), which may be due to changes in protein binding.

The $K_a$ values for azapropazone and warfarin are approximately the same ($5 \times 10^5$ and $3 \times 10^5$ M$^{-1}$, respectively), which makes it easy to understand that displacement reactions may occur when the two drugs are administered concomitantly. However, it is evident from Fig. 6 that drugs with lower association constants can effectively displace a drug which has a higher $K_a$ value. The figure indicates that the effect on drugs having high $K_a$ values and, consequently, a small free fraction will be relatively extensive. This is shown experimentally in Fig. 5, where naproxen is shown to be displaced by diazepam or flurbiprofen. The curves in Fig. 5 closely resemble the theoretical ones in Fig. 6, when the secondary binding is considered. As is evident, diazepam with a 10-fold lower $K_a$ effectively displaces naproxen.

The theoretical curves in Fig. 6 can also be used as models for the possible interaction between dicoumarol ($K_a = 3 \times 10^6$ M$^{-1}$; Ref. 13) and azapropazone ($K_a = 5 \times 10^5$ M$^{-1}$). Dicoumarol also binds to the warfarin site (6)

and is shown experimentally in Fig. 4 to be displaced by azapropazone. The low initial free concentration of dicoumarol renders the drug very sensitive for displacement phenomena, especially as dicoumarol has a low apparent distribution volume, $V_d$ (30). The clinical significance of the displacement will depend on the elimination mechanisms and on any dose-dependent elimination kinetics.

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