# General Treatment of Competitive Binding as Applied to the Potentiometric Ion Probe Technique: Application to the Interaction of Nonsteroidal Anti-Inflammatory Drugs with Bovine Serum Albumin

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**Abstract** □ The binding of naproxen, ketoprofen, phenylbutazone, salicyclic acid, azapropazone, and indobufen to bovine serum albumin was studied by applying the potentiometric ion probe technique. An ion-selective electrode for the ion probe 1-anilino-8-naphthalenesulfonate was utilized for the purposes of this study. A modified site-oriented competitive binding model was used for the estimation of the drugs' binding parameters, considering different number of binding sites on the competing binding class(es) for the probe and the drug. Calculations were based exclusively on the concentration data of the free probe. The model's ability for accurate estimations of binding parameters was evaluated by simulation studies. The following values of binding parameters were found at 25 °C for the drugs under study; naproxen, $n_1 = 9.1$, $k_1 = 9.4 \times 10^5$ M$^{-1}$; ketoprofen, $n_1 = 8.8$, $k_1 = 10.8 \times 10^5$ M$^{-1}$; phenylbutazone, $n_1 = 3.2$, $k_1 = 1.4 \times 10^5$ M$^{-1}$; salicylic acid, $n_1 = 2.6$, $k_1 = 1.8 \times 10^5$ M$^{-1}$, $n_2 = 21.5$, $k_2 = 1.0 \times 10^4$ M$^{-1}$; azapropazone, $n_1 = 0.5$, $k_1 = 7.8 \times 10^5$ M$^{-1}$, $n_2 = 26.3$, $k_2 = 1.9 \times 10^4$ M$^{-1}$; indobufen, $n_1 = 5.8$, $k_1 = 5.8 \times 10^5$ M$^{-1}$, $n_2 = 19.9$, $k_2 = 3.8 \times 10^5$ M$^{-1}$, where $n_i$ the number of binding sites of the $i$ class and $k_i$ the corresponding association constant.

Drug-protein binding has been known as an important factor in drug bioavailability, efficacy, transport, and toxicity. The reversible binding of a drug molecule to a protein is usually described by the site-oriented Scatchard model.[1] The major assumption in this model is that the binding sites on the protein molecule are classified to $m$ distinct and noninteracting classes, the $j$th class with $n_j$ identical binding sites having the same intrinsic binding constant, $k$. Alternatively, protein binding data can be analyzed with the stoichiometric binding model which is based on Klotz's equation.[2] In this case, the stoichiometric binding constants $K_x$ are calculated (for $x = 1$ to $Z$, where $Z$ is the maximal number of bound drug molecules).

Competitive binding phenomena resulting in displacement of drug from plasma proteins, are also known to play an important role in particular for drugs strongly bound to plasma proteins. Small changes in the percentage of binding of these drugs cause significant changes of their free plasma concentration, which is responsible for their pharmacological action. Protein binding equilibria involving two ligands are described with the stoichiometric model[3] or the site-oriented Scatchard model.[4,5]

A great number of experimental techniques have been applied to investigate the interaction of drugs with proteins. In the field of competitive binding where, usually, two ligands (the drug and the competitor) are competing for binding to the same binding sites on the protein molecule, the fluorescence probe technique,[6-8] has been widely used. The performance of this technique involves the displacement of a fluorescence probe bound to the protein by the drug under study. The assessment of the extent of the drug–protein binding is revealed by monitoring the change in the fluorescence intensity of the system. If the binding parameters of the probe are known, the parameters corresponding to the drug can be determined, provided that the drug and the probe share the same binding site on the protein.[6] Due to the empirical character of the calculations, the fluorescence probe studies are confined to displacement interactions involving only one class of binding sites. Circular dichroism (CD)[9] has also been used in competitive binding studies provided that the studied drugs have little or no measurable CD absorption in the wavelength region of the induced CD spectrum of the protein. Indirect techniques,[10] such as equilibrium dialysis, ultrafiltration, and gel filtration, have been also used. The main disadvantage of these techniques is the disturbance of the drug–protein equilibrium, caused by the separation of the free from the bound drug and the protein species.

In recent years considerable attention has been focused on the ion chemical sensors (ion-selective electrodes, ISEs) and their application to clinical[11] and pharmaceutical[12] analysis, as well as to binding studies.[13-18] ISEs are electrochemical transducers responding selectively, directly, and continuously to the free ion activity (concentration) in solution. The inherent advantage of ISEs, when applied to protein binding studies, is their ability to measure directly the concentration of the free ion of interest in the presence of the protein and the drug–protein complex. Moreover, ISE potentiometry is capable to measure the activity (concentration) of the free ion, with the same accuracy in dilute and concentrated protein solutions and moreover directly in biological specimens, overcoming thus the major drawbacks of the spectroscopic techniques.

Recently, we have developed[17] the potentiometric ion probe (PIP) technique for the study of sulfonamide–protein interactions using the model ion probe 1-anilino-8-naphthalenesulfonate (ANS). The technique is based on the continuous monitoring of the free ion probe concentration in the reaction mixture of protein–drug–probe, by direct potentiometry. However, the calculation of the association constants of drugs was accomplished[17] with the empirical relationships routinely used in fluorescence studies.[6] Accordingly, only estimates for the first class of binding sites were obtained.[17] In the present investigation, the ion probe ANS is utilized for the potentiometric study of the interaction of nonsteroidal anti-inflammatory drugs with bovine serum albumin (BSA) in conjunction with a new model developed. This model, which in reality is a modification of the site-oriented model, allows the estimation of the drug binding parameters (association constants and number of binding sites in each class) by nonlinear-regression analysis. The method is based on successive potentiometric measurements of the free probe concentrations which correspond to a series of known total probe concentrations in the reaction mixture of protein–drug–probe.

## Theoretical Section

**Model**—To illustrate the general approach, a competitive binding model, based on the classification of binding sites and equivalence between sites in each binding class, is considered

• Abstract published in *Advance ACS Abstracts*, May 15, 1994.



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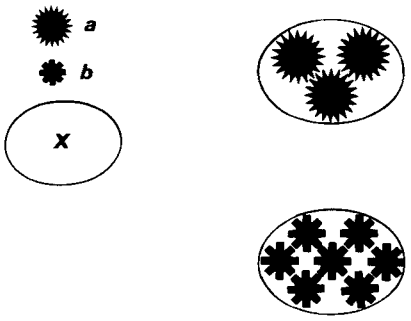


**Figure 1**—Schematic representation of the binding on two separate occasions, of two competing ligands to a class of binding sites on the protein molecule. Different numbers from each of the ligands a and b can bind to the area X (class of binding sites) depending on each ligand's structural characteristics.

for the drug (denoted with the subscript 1) and the probe (denoted with the subscript 2):[5]

$$r_1 = \sum_{j=1}^{m} \frac{n_{1j}k_{1j}F_1}{1 + k_{1j}F_1 + k_{2j}F_2} \quad (1)$$

$$r_2 = \sum_{j=1}^{m} \frac{n_{2j}k_{2j}F_2}{1 + k_{1j}F_1 + k_{2j}F_2} \quad (2)$$

where $r_i = B_i/P_t$ ($i$ = 1,2) is the number of moles of the $i$th ligand bound per mole of protein, $B_i$ and $F_i$ are the bound and free molar concentration of the $i$th ligand, respectively, $P_t$ is the total protein molar concentration, $n_{ij}$ is the number of binding sites for the $j$th class, with respect to the $i$th ligand, and $k_{ij}$ is the binding constant for the association of the $i$th ligand with the $j$th class of binding sites. This model has been used and accompanied[4,5] so far with the assumption of a rigid protein structure containing preformed independent binding sites. As a result of this, when the model is applied, the number of binding sites in each class is considered to be identical for both competing ligands, i.e. $n_{1j} = n_{2j}$. However, recent studies[19] have shown that a site may be formed upon binding of the ligand, and due to the flexibility of the protein molecules, the detailed formation of a binding site is dependent on the ligand's structure. It is reasonable therefore to infer that a different number of sites for a given class can be found for two competing ligands. This is schematically depicted in Figure 1.

**Simulation Studies**—Simulation studies were performed in order to evaluate the ability of the applied model (eqs 1 and 2) to describe competitive binding based on free probe concentration data without knowledge of the free drug concentration. In this context, simulated data were used for the evaluation of the model developed, in regard to its validity for the estimation of the binding constant. Moreover, simulation studies were undertaken to provide the forms of the Schatchard plots which are likely to be encountered in the experimental content.

Two ligands were considered, the probe with two classes of binding sites and the drug with one class of binding sites. Error-free simulated values for $r_1$ were calculated according to eqs 1 and 2, using a homemade program in BASIC. For this purpose, values were assigned for the parameters $n_{1j}$ and $k_{1j}$ (for $j$ = 1,2) and $n_{2j}$ and $k_{2j}$ (for $j$ = 1), while the $P_t$ concentration was set equal to $3.36 \times 10^{-4}$ M. A free probe concentration range ($F_1$) was also assigned ($1 \times 10^{-6} - 1 \times 10^{-3}$ M) and $r_1$ was calculated iteratively (Newton–Raphson algorithm). The free drug concentration $F_2$ for each pair of data ($r_1$, $F_1$) was calculated algebraically at each iteration for the calculation of $r_1$ from eq 3, which is obtained from eq 2, by replacing $r_2$ by its equal, $B_2/P_t = (T_2 - F_2)/P_t$:

$$F_2 = T_2 - \sum_{j=1}^{m} \frac{n_{2j}k_{2j}F_2}{1 + k_{1j}F_1 + k_{2j}F_2} P_t \quad (3)$$

where $T_2$ is the total drug concentration. Erroneous simulated data with various degrees of error were generated by adding to each error-free $F_1$ value a pseudorandom normal variate of mean zero and relative standard deviation equal to 1–10% of the error-free value. The error of the $F_1$ value is propagated to $r_1$, as the two variables (independent and dependent variable of eq 1, respectively) are correlated via the equation $T_1 = F_1 + B_1$ ($T_1$ is the total probe concentration), and therefore, $r_1 = B_1/P_t = (T_1 - F_1)/P_t$. Finally, eqs 1 and 3 were fitted to the generated data using the MINSQ[20] computer program, and an estimate of the drug's binding constant was obtained. Plots of the estimates for the drug's association binding constant versus the percentage of experimental error were constructed.

Scatchard plots ($r_1/F_1$ versus $r_1$) were also constructed for single- and multiple-class competition using simulated data. Two ligands were considered, the probe with three classes of binding sites and the drug with two classes of binding sites. Simulated data ($r_1$) were calculated according to eqs 1 and 3, using the MINSQ computer program. Values were assigned for the parameters, $n_{1j}$ and $k_{1j}$ (for $j$ = 1–3) and $n_{2j}$ and $k_{2j}$ (for $j$ = 1, 2); the $P_t$ concentration was set equal to $3.36 \times 10^{-4}$ M, while the $F_1$ range was also assigned ($1 \times 10^{-6}$–$1 \times 10^{-3}$ M). The free drug concentration $F_2$ for each pair of data ($r_1$, $F_1$) was calculated implicitly by the program from eqs 1 and 3, at each iteration for the calculation of $r_1$. The effect of the drug's binding capacity ($n_{21}k_{21}$) on the form of the probe's Scatchard curve, when competition occurs only for the first (high-affinity) class of binding sites of the probe, was also studied.

## Experimental Section

**Reagents**—All solutions were prepared in deionized water. 1-Anilino-8-naphthalenesulfonate ammonium salt (ANS) and bovine serum albumin fraction V were obtained from Fluka (Buchs, Switzerland). Naproxen, ketoprofen, phenylbutazone, salicylic acid, and azapropazone were kindly donated by local manufacturers. Indobufen was purchased from Farmitalia Carlo Erba. Phosphate buffer (0.1 M, pH 7.4) was used in the preparation of all solutions used. The ANS stock solution was 0.100 M and was used for the preparation of more-dilute ANS solutions. BSA solutions contained 22.5 g/L of protein, while mixed solutions contained also ANS, at a concentration of 0.0100 M and/or drug at various concentrations ($5 \times 10^{-4}$–$5 \times 10^{-3}$ M).

**Apparatus**—The ISE assembly and the preparation of the liquid ion exchanger were previously described.[14] The indicator electrode was used with an external Corning Ag/AgCl single-junction reference electrode filled with a 4 M KCl solution. The system used for the measurements has been previously described.[14,17] All measurements were performed at 25 ± 0.5 °C.

**Binding Experiments**—Binding experiments were performed using the ANS ion-selective electrode[14] to study the binding of six nonsteroidal anti-inflammatory drugs, namely salicylic acid, phenylbutazone, azapropazone, naproxen, ketoprofen, and indobufen with bovine serum albumin at pH 7.4.

**Procedures**—*Calibration Curve*—A 5.00-mL volume of phosphate buffer (0.1 M, pH 7.4) was pipetted into the measurement cell. The electrode were immersed in it, and after the potential was stabilized, various aliquots of a 0.0060 M ANS solution were added (concentration range covered $1.2 \times 10^{-6}$–$2 \times 10^{-3}$ M). The emf values were recorded and measured after stabilization (±0.1 mV) following each addition. The potential values, $E$, were plotted against $-\log C$ (p$C$) to give the calibration curve by linear-least-squares fitting. Corrections for the changes in volume after each addition were performed.

*Binding Experiments (Potentiometric Titration or Displacement Reaction)*—A 5.00-mL volume of the BSA solution, with or without drug, was pipetted into the measurement cell and the electrodes were immersed in it. After the potential was stabilized (±0.1 mV), small

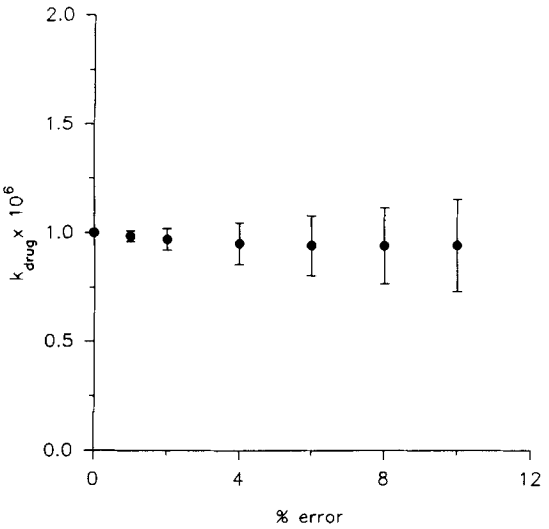


**Figure 2**—Plot of the means of the binding constant estimates versus the percent of the normally distributed error in the simulated data. The bar represents the standard deviation of the estimate. In all cases, the true value of $k_2$ was $1 \times 10^6$ $M^{-1}$.

amounts of the mixed ANS–BSA or the mixed ANS–BSA–drug solution, were added. The emf values were recorded to check stabilization (±0.1 mV) and measured after each addition. The concentration of both the drug and the protein remained constant by using the mixed titrant solutions.

**Data Analysis**—In our binding experiments, for every ANS addition, the free ANS concentration ($F_1$) was calculated from the calibration curve and the bound ANS concentration ($B_1$) from the difference $B_1 = T_1 - F_1$, where $T_1$ is the total ANS concentration. The binding parameters of the drug were estimated by nonlinear-least-squares simultaneous fitting of eq 1 and 3 to the experimental data ($r_1$, $F_1$) using the MINSQ nonlinear-least-squares fitting program. The free drug concentration ($F_2$) was treated as an implicit variable and was calculated either algebraically or numerically by a built-in root finder of the program for each iteraction involving $r_1$ and $F_1$. The binding parameters of the probe ($n_{1j}$, $k_{1j}$) were entered in the model as predetermined constant values; they were obtained from nonlinear-least-squares fitting[18] of the Scatchard model to the experimental data of a "blank" binding experiment, i.e. in the absence of drug. Three classes of binding sites were found for the ANS probe on BSA, the values of the binding parameters being as follows: $n_{11} = 2.7(\pm0.2)$, $n_{12} = 3.1(\pm0.2)$, $n_{13} = 15.0(\pm1.0)$, $k_{11} = 7.0(\pm0.8) \times 10^5$ $M^{-1}$, $k_{12} = 2.8(\pm0.1) \times 10^4$ $M^{-1}$, $k_{13} = 7.0(\pm0.2) \times 10^3$ $M^{-1}$.

The user of the program can select all possible types of interaction between the drug and the three binding classes of the probe, e.g. single competition for each one of the classes or multiple competition, specifying the classes and the order of intensity of the interaction. The program uses an extended Powell's algorithm for the least-squares fitting. Simplex procedure was applied for optimizing initial values for the least-squares procedure. Both the $F$-statistic and a modified Akaike model selection criterion (MSC) included in the MINSQ least-squares fitting program (the most appropriate model being that with the largest MSC) were used to evaluate differences between the various competition cases examined.

## Results and Discussion

**Simulation Studies**—Figure 2 shows a plot of the calculated values for the drug association constant versus the percent error of the simulated data. The almost-zero slope of the obtained linear curve reflects the ability of the used model to give accurate estimates for the binding constant even when significant error (10%) is included in the experimental data.

The Scatchard plots presented in Figure 3A illustrate the effect of the kind of competition on the probe's Scatchard curve. The significant changes observed can be indicative of the kind of competition. For example, competition of the drug for the high-affinity class of the probe on the protein molecule (even if

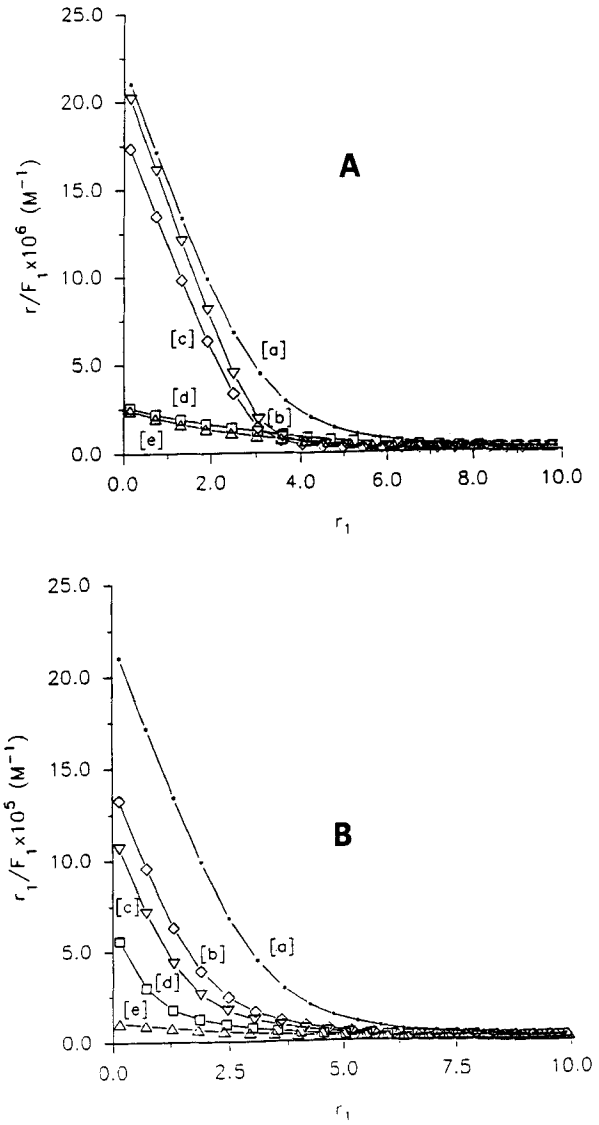


**Figure 3**—Analysis of simulated binding data. (A) Simulated probe Scatchard plots for different kinds of drug–probe competition assuming a probe with three classes and a drug with two classes of binding sites: a, in the absence of drug; b, primary competitive binding of the drug to a low-affinity and secondary to the high-affinity class of the probe; c, competitive binding of the drug to a low-affinity class of the probe; d, primary competitive binding of the drug to the high-affinity and secondary to a low-affinity class of the probe; e, competitive binding of the drug to the high-affinity class of the probe. In all cases, the following parameter values were assigned: $n_{11} = 2.5$, $n_{12} = 3$, $n_{13} = 15$, $k_{11} = 700\,000$ $M^{-1}$, $k_{12} = 30\,000$ $M^{-1}$, $k_{13} = 1000$ $M^{-1}$, $n_{21} = 1.5$, $n_{22} = 8$, $k_{21} = 75\,000$ $M^{-1}$, $k_{22} = 1000$ $M^{-1}$. (B) Effect of varying the drug's binding capacity ($n_{21}k_{21}$) on the form of the probe's Scatchard curve, assuming a probe with three classes and a drug with one class of binding sites. The drug is assumed to displace the probe from its high-affinity class of binding sites which has a binding capacity of $n_{11}k_{11} = 1.75 \times 10^6$ $M^{-1}$: a, in the absence of drug; b, $n_{21}k_{21} = 8 \times 10^6$ $M^{-1}$; c, $n_{21}k_{21} = 6 \times 10^6$ $M^{-1}$; d, $n_{21}k_{21} = 4 \times 10^6$ $M^{-1}$; e, $n_{21}k_{21} = 2 \times 10^6$ $M^{-1}$.

secondary competition also occurs for a low-affinity class of the probe) causes a dramatic reduction of the slope of the initial part of the probe's Scatchard curve (Figure 3A, cases d and e). On the other hand, primary competition of the drug for a low-affinity class of the probe (even if secondary competition also occurs for the high-affinity class of the probe) causes a parallel shift toward the $y$-axis of the probe's Scatchard curve (Figure 3A, cases b and c).

The effect of the drug's binding capacity (which corresponds

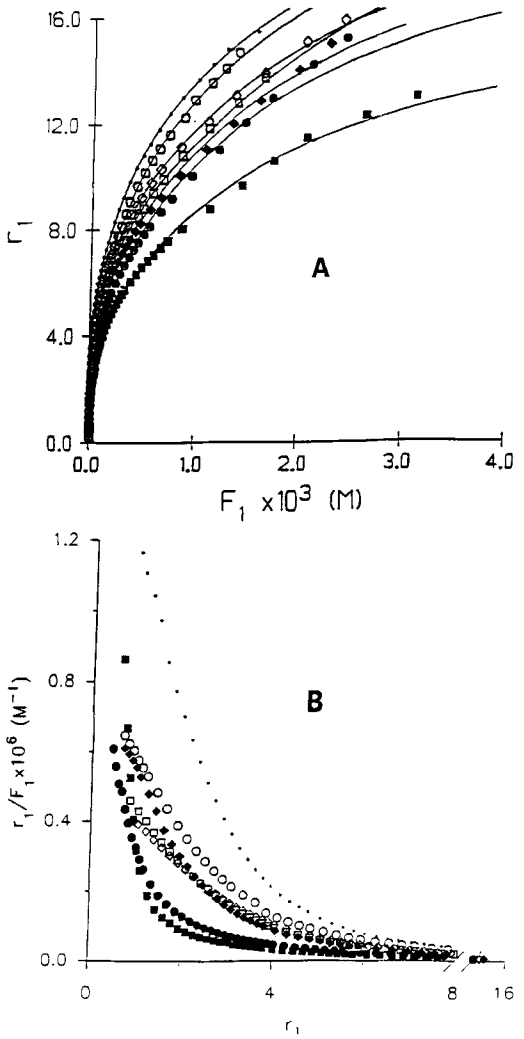


**Figure 4**—(A) Direct plots of the experimental data ($r_1$, $F_1$), along with the fitted lines based on eqs 1 and 3: ANS,*; salicylic acid, O; ketoprofen, ●; azapropazone, ◇; indobufen, ●; naproxen, ■. (B) The corresponding Scatchard plots.

to the product $n_{21}k_{21}$) on the form of the probe's Scatchard curve is illustrated in Figure 3B. This figure shows the most usual competition case, i.e. a drug with one class of binding sites competing for the high-affinity class of the probe. As it is shown in Figure 3B, the deviation of the probe's Scatchard curve from that in absence of drug becomes higher, when the binding capacity of the drug decreases. It is interesting to note the similarity of the plots in Figure 3B to plots b and c in Figure 3A, rather than to d and e. Therefore, conclusions concerning the type of competition as those derived from Figure 3A can lead to misinterpretations if the possibility of higher drug binding capacity with respect to the probe has not been ruled out.

**Binding Experiments**—The PIP technique was applied using the ion-selective electrode of ANS. Potentiometric data for the competitive binding of ANS with the nonsteroidal anti-inflammatory drugs, namely naproxen, ketoprofen, phenylbutazone, salicylic acid, azapropazone, and indobufen anions, were obtained. None of the studied drugs showed any significant interference with the ANS ISE in the range of ANS and drug concentrations used (an interference study was carried out using the mixed-solution method).

Plots of the experimental data ($r_1$ versus $F_1$) along with the fitted lines are shown in Figure 4A, and Figure 4B presents the corresponding Scatchard plots. The Scatchard plot of ketoprofen (Figure 4B) is similar to the plots in Figure 3B and represents

**Table 1—Estimates[a] for the Binding Parameters of the Drugs Obtained from Competitive Displacement Data**

| Drug | $n_1$ | $10^5k_1$ ($M^{-1}$) | $n_2$ | $10^4k_2$ ($M^{-1}$) | $R^2$ |
|---|---|---|---|---|---|
| Ketoprofen | 8.8(0.2) | 10.8(2.0) | | | 0.9990 |
| Naproxen | 9.1(0.3) | 9.4(2.2) | | | 0.9993 |
| Phenylbutazone | 3.2(0.1) | 1.4(0.2) | | | 0.9998 |
| Azapropazone | 0.5(0.7) | 7.8(2.6) | 26.3(3.2) | 1.9(0.4) | 0.9999 |
| Indobufen | 5.8(2.2) | 5.8(3.4) | 19.9(1.4) | 3.8(1.4) | 0.9997 |
| Salicylic acid | 2.6(1.1) | 1.8(0.6) | 21.5(3.1) | 1.0(0.2) | 0.9999 |

[a] Calculated at 25 °C, pH 7.4; within-run standard deviations of estimates in parentheses.

an example of a drug causing parallel shift toward the $y$-axis of the probe's Scatchard curve, because of its higher binding capacity.

Analysis of the experimental data with the model developed resulted in the estimates for the binding parameters listed in Table 1. The estimates for the drug-association constants are within the range of previously reported values.[21–25] For example, the association constants for the interaction of naproxen and azapropazone with human serum albumin (HSA) were found in the range $0.2 \times 10^5$–$1.1 \times 10^6$ $M^{-1}$ and 2.8–$9.1 \times 10^5$ $M^{-1}$, respectively.[21–23,25] Additionally, the number of classes of binding sites found for the studied drugs on BSA is in agreement with previous studies.[21–25] The model for single-competition (the high-affinity class of binding sites of ANS on BSA) yielded the best fit for naproxen, ketoprofen, and phenylbutazone. On the other hand, the model for multiple-competition (primary competitive binding for the high-affinity class and secondary for the second class of ANS) was found to be the most appropriate for azapropazone, indobufen, and salicylic acid. The within-run standard deviations of the binding estimates are excellent compared to the analogous measures of uncertainty derived from other protein binding techniques. Besides, the goodness of fit was reflected by the high values (0.9990–0.9999) for $R^2$ (Table 1).

It is worth noting that only salicylic acid showed a statistically equivalent value, $n_{21} = 2.6 \pm 1.1$, and phenylbutazone a relatively similar value, $n_{21} = 3.2 \pm 0.1$, to the number of binding sites of the first class, $n_{11} = 2.7$, assigned for ANS. For all other drugs listed in Table 1, the estimates for $n_{21}$ were significantly different from the value $n_{11} = 2.7$ assigned for ANS. These observations are in accord with our initial assumption that a different number of binding sites for the same class can be found for two competing ligands.

It should be mentioned here that phenylbutazone and azapropazone have been characterized as site I drugs while ketoprofen and naproxen are considered as site II drugs for HSA.[26] However, both site I and site II drugs were displaced from their sites on BSA by ANS. These results suggest that while the overall arrangement of sites on BSA and HSA is similar,[27] differences in the definition of individual sites occur. Site II on HSA and BSA may well involve different amino acid residues, and it can be expected that the site shows different conformational adaptability.[28] On the other hand, our results are also supported by the fact that ANS has not been proven to be a labeled Site I or Site II marker.[29]

The detectability of the method, i.e. the lower binding constant of a drug which can be determined accurately at a specified significance level, is limited by the combination of the sensitivity and detectability of the ion-selective electrode, the value and the precision of measurement of the binding constant of the probe (ANS), and of course the concentration of the drug used. The detection limit of a drug's binding constant can be defined as that value which apparently decreases the first class binding constant of the probe to its lower confidence limit (95% significance level). By using simulated data and assigning a

value of $k_1 - 2SD = 7.0 \times 10^5 - (2 \times 0.8 \times 10^5) = 5.4 \times 10^5$ $M^{-1}$ for the lower confidence limit of the ANS binding constant, the detection limit for the drug binding constants were found to be 150 and 300 $M^{-1}$ for total drug concentration of $1 \times 10^{-3}$ and $5 \times 10^{-3}$ M, respectively.

In theory, the model developed can be applied directly to the experimental displacement data for the simultaneous estimation of the binding parameters of both ligands, i.e. drug and probe. In the present study, the probe binding constants, obtained from separate experiments, were introduced as adjustable parameters in the program, in order to reduce the variance of the drug binding parameter estimates. Plausibly, the model can be also used if free concentration data for both ligands (probe and drug) are available. However, the large number of experimental data points (the inherent advantage of the PIP technique) allowed the estimation of the drug binding parameters without the knowledge of the free drug concentration. The latter was calculated implicitly by the computer program utilized.

This study extents the utility of direct potentiometry for displacement phenomena when the two ligands (drug and probe) exhibit different numbers of binding sites for each class of the protein. The model developed can be used to evaluate the drug's binding parameters, provided that a probe sharing the same classes of binding sites with the drug is available. Needless to say, the model can be also utilized with other techniques if valid free probe concentrations can be obtained. This approach offers a valuable and rapid tool of analysis for displacement phenomena based on the current views either of the flexibility of binding sites[19] or the conceptually relevant partial competition[30] of competitive binding assays. The present demonstration of the feasibility of monitoring the entire profile of the ANS–BSA interaction in the presence of anti-inflammatory drugs justifies the potential use of this technique for the study of the displacement phenomena directly in biological specimens.

## Abbreviations

Circular dichromism, CD; ion selective electrodes, ISEs; potentiometric ion probe, PIP; 1-anilino-8-naphthalenesulfonate, ANS; bovine serum albumin, BSA; human serum albumin, HSA; model selection criterion, MSC.

## References and Notes


1. Scatchard, G. *Ann. N. Y. Acad. Sci.* **1949**, *51*, 660–672.
2. Klotz, I. M. *Arch. Biochem.* **1946**, *9*, 109–117.
3. Honore, B.; Brodersen, R. *Mol. Pharmacol.* **1983**, *25*, 137–150.
4. Feldman, H. N. *Anal. Biochem.* **1972**, *48*, 317–338.
5. Veng Pedersen, P. *J. Pharm. Sci.* **1978**, *67*, 908–913.
6. Essassi, D.; Zini, R.; Tillement, J.-P. *J. Pharm. Sci.* **1990**, *79*, 9–13.
7. Sato, J.; Sugiyama, Y.; Hanano, M. *J. Pharm. Sci.* **1984**, *73*, 519–524.
8. Hsu, P.-L.; Ma, J. K. H.; Jun, H. W.; Luzzi, L. A. *J. Pharm. Sci.* **1974**, *63*, 27–31.
9. Watanabe, S.; Saito, T. *Biochem. Pharmacol.* **1992**, *43*, 931–935.
10. Pacifici, G.-M.; Viani, A. *Clin. Pharmacokinet.* **1992**, *23*, 449–468.
11. Diamandis, E. P.; Koupparis, M. A.; Hadjiioannou, T. P. *Microchem. J.* **1977**, *22*, 498–504.
12. Campanella, L.; Tomassetti, M. *Selective Electrode Rev.*, **1989**, *11*, 69–110.
13. Valsami, G. N.; Macheras, P. E.; Koupparis, M. A. *J. Pharm. Sci.* **1990**, *79*, 1087–1094.
14. Valsami, G. N.; Macheras, P. E.; Koupparis, M. A. *Pharm. Res.* **1991**, *8*, 888–892.
15. Valsami, G. N.; Koupparis, M. A.; Macheras, P. E. *Pharm. Res.* **1992**, *9*, 94–100.
16. Sideris, E. E.; Valsami, G. N.; Koupparis, M. A.; Macheras, P. E. *Pharm. Res.* **1992**, *9*, 1568–1574.
17. Angelakou, A.; Valsami, G.; Koupparis, M.; Macheras, P. *J. Pharm. Pharmacol.* **1993**, *45*, 434–438.
18. Christopoulos, T.; Diamandis, E. *Anal. Chem.* **1990**, *62*, 360–367.
19. Honore, B. *Pharmacol. Toxicol.* **1990**, *667* (Suppl.II), 1–26.
20. *Minsq Non-linear Parameter Estimation and Model Development*, User Handbook, MicroMath Scientific Software, 1992.
21. Kragh-Hansen, U. *Mol. Pharmacol.* **1988**, *34*, 160–171.
22. Li, K. L.; Thakur, A. K.; Kapoor, A. L. *J. Pharm. Sci.* **1988**, *77*, 251–254.
23. Diana, F. J.; Veronich, K.; Kapoor, A. L. *J. Pharm. Sci.* **1989**, *78*, 195–199.
24. Lapicque, F.; Muller, N.; Payan, E.; Dubois, N.; Netter, P. *Clin. Pharmacokinet.* **1993**, *25*, 115–125.
25. Lin, J. H.; Cocchetto, D. M.; Duggan, D. E. *Clin. Pharmacokinet.* **1987**, *12*, 402–432.
26. Sjohom, I.; Ekman, B.; Kober, A.; Ljungsted-Pahlman, I.; Seiving, B.; Sjodin, J. *Mol. Pharmacol.* **1979**, *16*, 767–777.
27. Panjehshahin, M. R.; Yates, M. S.; Bowmer, C. J. *Biochem. Pharmacol.* **1992**, *44*, 873–879.
28. Walji, F. R. Ph.D. Thesis, University of London, 1990.
29. Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1975**, *11*, 824–832.
30. Brocklebank, A. M.; Sawyer, W. H.; Wiley, J. S.; Winzor, D. J. *Anal. Biochem.* **1993**, *213*, 104–110.