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A displacement approach for competitive drug–protein binding studies using the potentiometric 1-anilino-8-naphthalene-sulfonate probe technique

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Abstract

A displacement approach for competitive binding studies was developed. The method utilizes the potentiometric 1-anilino-8-naphthalene-sulfonate (ANS) probe technique and is applied to the binding study of several non-steroidal anti-inflammatory drugs (NSADs) to bovine serum albumin (BSA). A home-made ANS electrode was used to monitor the displaced free ANS probe from its binding sites on the protein molecule by the stepwise addition of the studied drug. To assess and compare quantitatively the displacing ability of the various drugs, the 'ANS Displacement Index' is used. The possible interference of 19 ionizable drugs (NSADs, sulfonamides, etc.) to the ANS selective electrode at pH 7.4 was studied and their potentiometric selectivity coefficients ($K^{pot}_{ANS,D}$) were determined. Correction procedures for the determination of the free ANS concentration are proposed in the case of interfering ionic drugs. A blank binding experiment in conjunction with the incorporation of $K^{pot}_{ANS,D}$ values in the 'general competitive site oriented model' allows one to derive estimates for the drug binding parameters, i.e. the number of binding sites and association constants.

Keywords: Protein binding; Non-steroidal anti-inflammatory drugs; 1-Anilino-8-naphthalene-sulphonate; Ion selective electrodes; Potentiometric selectivity coefficient; Potentiometric probe technique

1. Introduction

Drug–protein binding studies are of great importance in pharmaceutical sciences and several techniques have been developed and applied for the determination of the binding parameters. 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 (Sudlow et al., 1975; Essassi et al., 1990; Bagattoli et al., 1996) has been widely used. The performance of the technique involves the displacement of a fluorescent probe bound to the protein by the drug under study. The assessment of the extent of 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 sites on the protein (Sudlow et al., 1975; Essassi et al., 1990; Bagattoli et al., 1996). The main disadvantage of the technique is the inability of using it for colored and concentrated protein solutions. Circular dichroism (CD) (Watanabe and Saito, 1992) 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, such as equilibrium dialysis, ultrafiltration, and gel filtration (Vallner, 1977; Pacifici and Viani, 1992), have also been 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.

One of the most powerful techniques developed during the last decade for binding studies of ionic micromolecules (e.g., drugs) to macromolecules (proteins, cyclodextrins) is direct potentiometry using ion sensors (electrodes) selective to the ionic micromolecule (Takisawa et al., 1988; Christopoulos and Diamandis, 1990; Valsami et al., 1990, 1991, 1992; Sideris et al., 1992). The technique involves titration of the macromolecule (e.g., protein) with the studied drug solution and direct measurement of the

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electrode potential which is a function of the activity (concentration) of the free ionic drug. Using a calibration (response) curve of the electrode the free drug concentration can be calculated and used for the determination of the binding parameters according to either the stoichiometric or the site oriented model. The inherent advantage of the technique is the direct measurement of the concentration of the free drug over a wide concentration range in the presence of the protein and their complexes and thus provides a complete binding profile. The main limitations of the technique are the necessity of a successful ion sensor for each drug of interest and its applicability only to the ionic species of the drug. In order to overcome these two drawbacks, our group developed the potentiometric ion probe technique, in which the binding of water-soluble drug (ionic or non-ionic) to a protein can be monitored by measuring potentiometrically a competitive ion probe (Angelakou et al., 1993, 1994). As a probe, 1-anilino-8-naphthalenesulfonate (ANS) has been used and a very successful ion selective electrode (ISE) has been constructed (Valsami et al., 1991; Angelakou et al., 1993, 1994). The potentiometric 'titration' methodology using ANS probe ISE involves simple microtitration procedures of 15–30 min each, providing a significant number of experimental data. In comparison with indirect techniques, e.g. dialysis, or direct techniques requiring the preparation of a batch of mixtures for spectrophotometric or fluorimetric measurements, the method is simple and fast. In the case of ionic drugs, the success of this technique depends on the absence of any serious interference of the studied drug ion on the probe electrode. ISEs are not specific sensors and chemically similar ions can interfere with the electrode's response through an ion-exchange process on the electroactive membrane of the ISE.

So far, the ANS probe technique has been performed using the so-called 'inhibition approach', i.e. titration of a protein–drug solution with ANS (Angelakou et al., 1993, 1994). In this work, we develop the potentiometric probe displacement approach. The ANS electrode is used to monitor the displaced free ANS probe from its binding sites on the protein molecule by the stepwise addition of the studied drug. This approach allows us to propose for the first time the 'ANS Displacement Index' as a quantitative indicator for the competitive binding of each of the studied drugs. We also studied the possible interference of 19 drugs (NSADs, sulfonamides, etc. with ionizable acidic groups) on the ANS electrode and developed correction procedures for both the displacement and inhibition approaches.

2. Experimental

2.1. Apparatus

The ANS electrode is of the PVC type, developed in our laboratory, with an electroactive ion-exchanger consisting of the ANS–tetraheptylammonium ion pair in *p*-nitrocumene. Its construction and characteristics have been described in detail elsewhere (Valsami et al., 1991). A Ag/AgCl single junction reference electrode was used to complete the electrochemical cell. An Orion (Model 801) digital electrometer (readability ± 0.1 mV) connected to a chart-recorder was used for the potential measurements. All experiments were carried out in a double wall cell thermostated at 25°C. Additions of microvolumes to the monitored solution in the cell were achieved accurately using Hamilton microsyringes.

2.2. Reagents

1-Anilino-8-naphthalenesulfonate ammonium salt and bovine serum albumin (BSA, fraction V, 96–99%) were from Fluka (Buchs, Switzerland). The studied drugs were kindly provided or purchased from the following manufacturers or suppliers: ketoprofen and azapropazone (Selectchemie AG), tolmetin sodium dihydrate (Cillag AG), ibuprofen (Boots) naproxen (Syntex Research), warfarin (Chemoswed AB; a subsidiary of Ferrosan, Sweden), sodium salicylate (Ferak), tolbutamide and diflunisal (Sigma), phenylbutazone (Adelco), iopanoic acid (Sterling Organics), suprofen (Janssen Res. Foundation), indobufen (Farmitalia Carlo Erba), l-tryptophan (BDH Chemicals Ltd.), sulfamethizole, sulfamethoxazole, sulfadiazine and sulfisoxazole (sulfafurazole) (American Cyanamid Company) and indomethacin (Merck).

All solutions were prepared in phosphate buffer, 0.10 M, pH 7.4. The ANS stock solution was 0.010 M. Drug stock solutions in the range 0.010–0.10 M were prepared by dissolving the appropriate amount in buffer or in an equivalent amount of NaOH and then diluted in the buffer. The BSA solution was 22.5 g/l (3.36×10^{-4} M) and was stored at 4°C for no more than 1 week. Mixed ANS–BSA, ANS–drug and ANS–drug–BSA solutions were also prepared.

3. Procedures

3.1. Construction of the calibration curve of the ANS ISE

The pair of electrodes was immersed in 3 ml phosphate buffer, 0.10 M, pH 7.4. After the potential was stabilised (± 0.1 mV), small volumes of a 6×10^{-3} M ANS solution were added (concentration range studied 1.3×10^{-6} to 2.4×10^{-3} M). The potential values (mV) were recorded and measured after stabilisation (± 0.1 mV), following each addition. These were plotted against log C_{ANS} according to the Nernst equation to obtain the calibration curve and the corresponding equation using linear least-squares

fitting. The calibration curve was constructed before each binding experiment.

3.2. Interference study of the drugs to ANS ISE

The experiments were carried out according to the mixed solution approach (Moody and Thomas, 1970). The pair of electrodes was immersed in 3 ml of a solution of the drug under study of concentration C_D (range 5×10^{-4} to 5×10^{-3} M) prepared in phosphate buffer, 0.10 M, pH 7.4. After the potential was stabilised (±0.1 mV), microvolumes of an ANS (6×10^{-3} M)–drug (C_D) mixed solution were added. The total ANS concentration range covered was exactly the same as in the construction of the calibration curve experiments (1.3×10^{-6} to 2.4×10^{-3} M), while the total drug concentration (C_D) remained constant. The experimental potential and free ANS concentration in the presence of drug were further used to determine the potentiometric selectivity coefficient ($K^{pot}_{ANS,D}$) of the studied interfering drug ion as described in Results and discussion.

3.3. Binding experiments

3.3.1. Displacement experiment

Three millilitres of a mixed ANS (2×10^{-4} M)–BSA (3.36×10^{-4} M) solution in phosphate buffer, 0.10 M, pH 7.4, was titrated stepwise by micro-volume addition of a mixed ANS (2×10^{-4} M)–BSA (3.36×10^{-4} M)–drug solution in order to keep the ANS and BSA concentration constant during the binding experiment, while the total drug concentration ranged from 3×10^{-5} to 8×10^{-3} M. The potential values measured were used to calculate the free ANS concentration from the calibration curve.

3.3.2. Blank experiment (correction of the displacement experiment)

Three millilitres of a BSA (3.36×10^{-4} M) solution were titrated with a mixed BSA (3.36×10^{-4} M)–drug solution in order to keep the BSA concentration constant during the blank experiment, while the total drug concentration was changed in the same range as in the binding experiment (3×10^{-5} to 8×10^{-3} M). The experimental data were used to calculate the possible interference (absolute positive error of the free ANS concentration) due to the presence of the interfering drug.

3.3.3. Inhibition experiment

Three millilitres of a BSA or a mixed BSA (3.36×10^{-4} M)–drug solution was titrated with a mixed ANS (0.010 M)–BSA (3.36×10^{-4} M) or ANS (0.010 M)–BSA (3.36×10^{-4} M)–drug (5×10^{-4} to 1×10^{-3} M) solution, in order to keep the BSA, or drug and BSA, concentration constant during the binding experiment, while the ANS concentration ranged from 1.0×10^{-6} to 7.5×10^{-3} M.

4. Results and discussion

4.1. Interference study of the drugs on the ANS ISE

It is well known that an ISE for a primary ion, X, may also respond to another chemically similar (interfering) ion, Y, causing a positive error in the determination of the primary ion concentration. As an example, the response curve of the ANS electrode in the absence and presence of the ionic drug diflunisal is shown in Fig. 1. Diflunisal was found to interfere with the ANS electrode when used at a constant concentration, $C_D = 5\times10^{-4}$ M. As can be seen, the interference caused serious relative errors at low ANS concentrations.

The experimental data from interference studies can be analysed according to the Nicolsky (1937) equation (Eq. (1)) to quantify the interfering effect of a drug on the ANS electrode:

$$E = E_{const.} + S\log([X] + K^{pot}_{X,Y}[Y]^{Z_X/Z_Y}) \quad (1)$$

where E is the electromotive force of the electrochemical cell (see Section 2.1), E_{const} is a constant term, S is the slope of the electrode, Z_X and Z_Y are the charges of the primary (X) and the interfering (Y) ion, respectively, and $K^{pot}_{X,Y}$ is the so-called potentiometric selectivity coefficient. The percent relative error for the determination of the primary ion concentration (concentration can be used instead of activity when constant ionic strength is used) is given by the equation

$$\%\ \text{rel. err.} = \frac{K^{pot}_{X,Y}[Y]^{Z_X/Z_Y}}{[X]}\cdot 100 \quad (2)$$

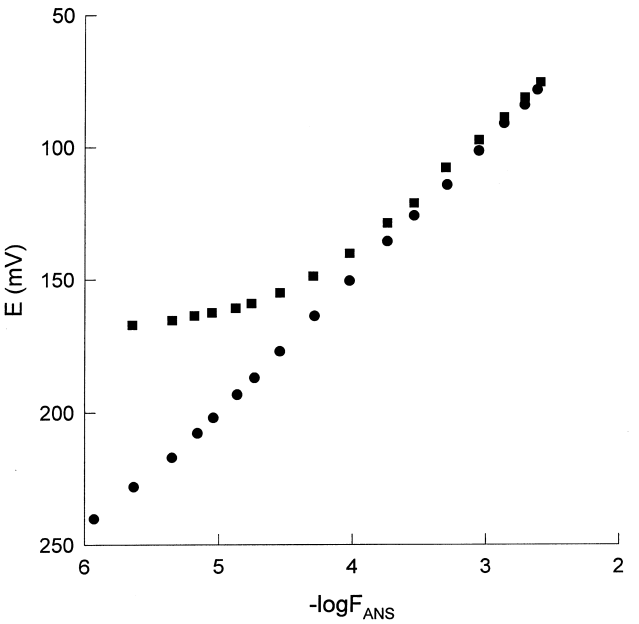

Fig. 1. Calibration curves of ANS ISE in the absence (●) and presence (■) of diflunisal, 5×10^{-4} M.

For our experiments, $Z_{ANS} = Z_D = -1$; thus, Eq. (1) can be written

$$E_i = E_{const.} - S \log[C_{ANS(i)} + K^{pot}_{ANS,D} C_D] \quad (3)$$

where the values for $E_{const.}$ and S are known from the calibration curve of the electrode and C_D is the constant drug concentration for the interference study. Therefore, Eq. (3) can be used to derive an estimate for $K^{pot}_{ANS,D}$ by measuring the potential of the electrode E_i for various ANS concentrations (ANS_i) in the presence of a constant concentration of the drug, C_D (procedure 3.2).

Table 1 summarizes the results of the interference study for 19 selected drugs. These drugs mainly belong to the non-steroidal anti-inflammatory drugs (with –COOH as ionisable functional group), the sulfonamides (with $-SO_2NH_2$ as ionisable group) and the remainder possess the –OH enolic group. From the pK_a values listed in Table 1 it is clear that are fully ionized at pH 7.4. The $K^{pot}_{ANS,D}$estimates quoted in Table 1 were derived from the non-linear least-squares fitting of Eq. (3) to the experimental data. The relatively low values of within-run %RSD found (3–18%, Table 1) indicate that the model (Eq. (3)) is valid for the description of data within the concentration ratio C_D/C_{ANS} range used. None of the drugs studied showed extremely high interference, $K^{pot}_{ANS,D} \geq 1$, the higher ones being diflunisal, warfarin and phenylbutazone ($K^{pot}_{ANS,D} = 0.116$–0.024). The interfering effect of some drugs was studied at two drug concentrations in order to examine the validity of the approach in estimating $K^{pot}_{ANS,D}$ at various concentrations, C_D. As shown in Table 1, the $K^{pot}_{ANS,D}$ values for phenylbutazone, ketoprofen and iopanoate remained practically constant at the two concentrations studied.

Table 1
Potentiometric selectivity coefficients of various drug anions towards the ANS electrode at 25°C and pH 7.4. ANS molar concentration ranged from 1.3×10^{-6} to 2.4×10^{-3} M

Drug, functional group/pK_a	Drug conc. ($\times10^{-3}$ M)	$K^{pot}_{ANS,D}$ ($\times10^{-3}$)[a]
Ibuprofen –COOH/5.2	2.0	2.2 (0.3)
Naproxen –COOH/4.15	2.0	1.1 (0.1)
Suprofen –COOH/3.91	1.0	4.8 (0.4)
Ketoprofen –COOH/4.0	2.0	3.4 (0.6)
	5.0	1.0 (0.1)
Indobufen –COOH/–[b]	2.0	1.34 (0.04)
Salicylate –COOH/2.97	1.0	2.0 (0.3)
Diflunisal –COOH/3.30	0.5	116 (6.0)
Indomethacin –COOH/4.5	2.0	0.94 (0.09)
Azapropazone –OH(enolic)/6.3	2.0	3.44 (0.16)
Phenylbutazone –OH(enolic)/4.5	0.5	24.0 (1.0)
	2.0	20.0 (0.6)
Tolmetin –COOH/3.5	5.0	0.34 (0.09)
Tolbutamide $-SO_2NH-$/5.43	2.0	1.76 (0.16)
Warfarin –OH(enolic)/5.05	2.0	42.0 (3.0)
L-Tryptophan –COOH/2.38	2.0	2.69 (0.51)
Iopanoate –COOH/4.8	0.5	7.24 (0.34)
	1.0	13.0 (1.0)
Sulfamethoxazole $-SO_2NH-$/5.6	1.0	0.31 (0.04)
Sulfadiazine $-SO_2NH-$/6.48	0.5	0.43 (0.05)
Sulfafisoxazole $-SO_2NH-$/5.0	0.5	0.75 (0.06)
Sulfamethizole $-SO_2NH-$/5.45	0.5	0.27 (0.03)

[a] Numbers in parentheses are within-run standard deviations of the estimates.
[b] Not reported.

The decision for the need of a correction step in a binding study depends on the $K^{pot}_{ANS,D}$ value in conjunction with the maximum drug concentration utilized. For example, a 50% allowable maximum relative error at the quantitation limit of the ANS probe ($\approx1\times10^{-6}$ M) and a maximum free drug concentration of 1×10^{-3} M (Eq. (2)), produces 5×10^{-4} M as the maximum allowable value for $K^{pot}_{ANS,D}$. This maximum allowable error corresponds to an absolute error of 5×10^{-7} M and a relative error ranging from 50 to 0.0066% for the ANS working range 1×10^{-6}–7.5×10^{-3} M. Therefore, ionic drugs with $K^{pot}_{ANS,D}$values greater than this limit require a correction step in the binding study. Most of the drugs listed in Table 1 lie above this limit. However, one should keep in mind that this high relative error can occur near the quantitation limit of the ANS electrode.

4.2. Displacement experiment – correction procedure

In the displacement experiment the increasing drug concentration causes a stepwise displacement of the ANS from its binding sites on the protein molecule. The values of free ANS concentration (F_{ANS}) are subject to positive error which can be considered proportional to the product $K^{pot}_{ANS,D} \times F_{D(i)}$, where $F_{D(i)}$ is the free drug concentration. Due to the continuous increase of $F_{D(i),}$ the error in the potential readings of ANS increases in a complicated manner during the displacement experiment. This realization prompted us to design the following simple correction procedure. The main binding displacement experiment is followed by a blank titration of BSA with the interfering drug in the absence of ANS. Since $F_{ANS} = 0$, it is clear from Eq. (3) that the E_i value readings of the blank titration correspond (through the calibration curve of the electrode) to the quantity $K^{pot}_{ANS,D} \times F_{D,blank(i)}$. Assuming that the presence of the ANS probe in the displacement

experiment does not increase $F_{D(i)}$ to a considerable degree (ANS is used at a total concentration of 2×10^{-4} M and is mostly bound to the protein) the measured 'apparent ANS concentration' at each point of the blank titration, $F_{ANS,blank(i)}$, can be considered equal to the positive error caused by the presence of $F_{D(i)}$ in the real displacement experiment. Hence, the free ANS concentrations, $F_{ANS(i)}$, of the displacement experiment was calculated from

$$F_{ANS(i)} = F_{ANS,appar.(i)} - F_{ANS,blank(i)} \quad (4)$$

The displacement experiment and the described correction procedure for drug interference on the ANS electrode were used for the binding study of various NSADs (ibuprofen, naproxen, ketoprofen, suprofen, indobufen, tolmetin, salicylic acid, azapropazone, phenylbutazone) and iopanoic acid with BSA. Selection of the drugs was based on their high binding affinity for BSA from previous studies (Angelakou et al., 1994). Fig. 2 shows typical plots ($F_{ANS(i)}$ vs. $C_{D(i)}$) for the binding study of azapropazone to BSA using the ANS displacement technique, before and after correction for azapropazone interference on the ANS electrode. In Fig. 3 the ANS displacement curves ($\%B_i/B_0$ vs. $C_{D(i)}$) for all the drugs studied are shown.

It is well known that competitive protein binding depends on the molar concentration ratio of the competing molecules as well as the drug/protein molar ratio. In our experiments the total molar concentration drug/BSA (3.36×10^{-4} M) ratio was $\geq$6:1, while the corresponding drug/ANS (2×10^{-4} M) ratio was $\geq$10:1. Under these experimental conditions all studied drugs displaced ANS from its binding sites on the BSA molecule to various degrees. This means that even the drugs with the weaker binding affinities for BSA induced a measurable free ANS concentration. When displacement occurs at the lower drug/BSA (6:1) and drug/ANS (10:1) concentration ratios, ANS is most likely displaced from its primary class of binding sites on the BSA molecule. On the other hand, drugs which do not compete with ANS for its primary class of binding sites cause negligible ANS displacement at low drug concentration ($\leq2\times10^{-3}$ M). However, when these drugs are used at higher concentrations, ANS displacement is observed, which is probably due to conformational changes of the BSA molecule affecting ANS affinity for BSA. Similar behavior has also been reported for the action of elevated free fatty acids (FFT) concentration on the protein binding of some drugs (Santos and Spector, 1974). ANS binding was not changed for molar concentration FFT/HSA ratios of 1 or 2, but when this ratio became >3 significant ANS displacement from its binding sites was observed (Santos and Spector, 1974). In the same vein, the competitive binding of site I bound drugs on the HSA molecule by site I probes for a 1:1 drug/probe molar concentration ratio has also been described (Wilding et al., 1977). However, for the same drug/probe concentration ratio, site II bound drugs are not displaced from their binding sites on the HSA molecule. When higher drug/protein concentration ratios are used, both site I and site II bound drugs are displaced from their binding sites on the HSA molecule (Wilding et al., 1977).

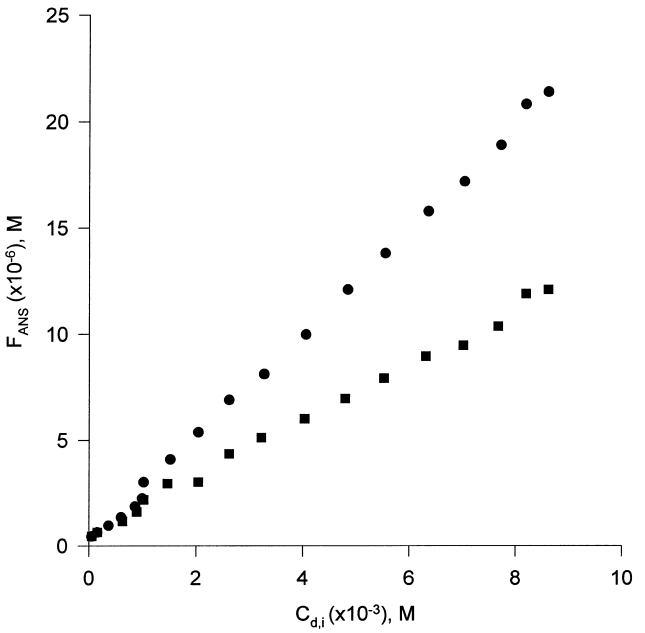

Fig. 2. Free ANS concentration change in relation to the total concentration of azapropazone before (●) and after (■) correction for interference according to Eq. (4): $F_{ANS(i)} = F_{ANS,appar.(i)} - F_{ANS,blank(i)}$.

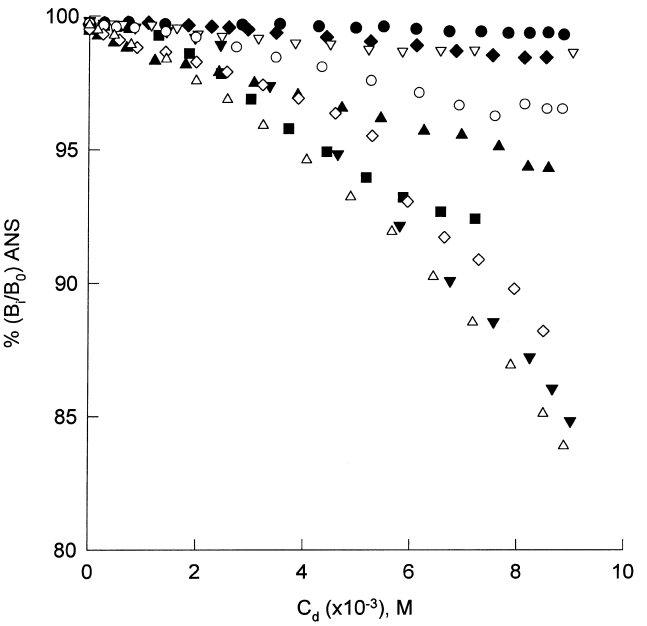

Fig. 3. ANS displacement curves for all drugs studied: tolmetine (●), naproxen (♦), salicylate (▽), ketoprofen (○), azapropazone (▲), indobufen (◊), iopanoate (■), phenylbutazone (▼), and ibuprofen (△). B_i is the bound ANS concentration at each total drug concentration, and B_0 is the bound ANS concentration at the beginning of the displacement experiment in the presence of drug (BSA concentration 3.36×10^{-4} M).

The displacement experiments can also be evaluated in a comparative manner to quantify the potential for ANS displacement of the various drugs. To this end, the ANS Displacement Index at the 1% level of displacement ($ADI_{1\%}$) was used:

$$ADI_{1\%} = \frac{B_0}{C_{D,1\%} C_{ANS}} \cdot \frac{1}{100} \quad (5)$$

where $C_{D,1\%}$ is the total drug concentration causing a 1% ANS displacement from its binding sites on the protein molecule, B_0 is the bound ANS molar concentration in the absence of drug (at the beginning of the binding experiment) and C_{ANS} is the total ANS concentration used. Table 2 presents the calculated $ADI_{1\%}$ values. The drug with the highest $ADI_{1\%}$ value is the drug showing the highest ANS displacement from its binding sites on the BSA molecule. Azapropazone displaced 1% ANS ($ADI_{1\%} = 9.78$) at drug/BSA and drug/ANS concentration ratios of 3 and 5.1, respectively, followed by phenylbutazone and indobufen ($ADI_{1\%} = 8.32$), which caused the same ANS displacement when the above ratios were 3.6 and 6, respectively. This behaviour is indicative of the competition of these drugs with ANS for the primary class of binding sites of the probe on the BSA molecule. The smaller $ADI_{1\%}$ values calculated for the other drugs studied reflect their lower ability to displace 1% of ANS.

4.3. Inhibition experiment – correction procedure

In the inhibition experiment the free ANS concentration, F, calculated from the calibration curve of the electrode for each point of the binding titration experiment, is used for the determination of the drug (inhibitor) binding parameters using the general competitive binding model (Angelakou et al., 1994). The model, based on the site-oriented Scatchard (1949) model, i.e. classification of binding sites and equivalence between sites in each class for the probe (denoted by subscript 1) and the drug (denoted by subscript 2), is described by the equations

$$r_1 = \sum_{j=1}^{m} \frac{n_{1j} k_{1j} F_1}{1 + k_{1j} F_1 + k_{2j} F_2} \quad (6)$$

$$r_2 = \sum_{j=1}^{m} \frac{n_{2j} k_{2j} F_2}{1 + k_{1j} F_1 + k_{2j} F_2} \quad (7)$$

replacing r_2 in Eq. (7) with $r_2 = B_2/P_t = (T_2 - F_2)/P_t$ and solving in terms of F_2:

$$F_2 = T_2 - \sum_{j=1}^{m} \frac{n_{2j} k_{2j} F_2}{1 + k_{1j} F_1 + k_{2j} F_2} P_t \quad (8)$$

where $r_i = B_i/P_t$ is the number of moles of the ith ligand bound per mole of protein, B_i, F_i and T_i are, respectively, the bound, free and total molar concentration of the ith ligand, P_t is the total protein molar concentration, n_{ij} is the number of binding sites for the jth class with respect to the ith ligand, and k_{ij} is the binding constant for the association of the ith ligand with the jth class of binding sites (for $i = 1,2$). In the presence of an interfering ionized drug, the measured values of free ANS concentration, $F_{1,appar.}$, are higher than the real F_1 values by a value equal to the positive error caused by the interfering ionized drug, i.e. by the product $K^{pot}_{1,2} \times F_2$, where F_2 is the free drug concentration at each point of the binding experiment. Therefore,

$$F_1 = F_{1,appar.} - [K^{pot}_{1,2} F_2] \quad (9)$$

The values of F_2 vary from a minimum (at the commencement of the binding experiment) to a maximum value which is almost equal to the total drug concentration, T_2 (when the drug is totally displaced by the probe). Therefore, the general competitive model described by Eqs. (6) and (8) was modified to take into account the correction for F_1:

$$r_1 = \sum_{j=1}^{m} \frac{n_{1j} k_{1j} (F_{1,appar.} - K^{pot}_{ANS,D} F_2)}{1 + k_{1j} (F_{1,appar.} - K^{pot}_{ANS,D} F_2) + k_{2j} F_2} \quad (10)$$

$$F_2 = T_2 - \sum_{j=1}^{m} \frac{n_{2j} k_{2j} F_2}{1 + k_{1j} (F_{1,appar.} - K^{pot}_{ANS,D} F_2) + k_{2j} F_2} P_t \quad (11)$$

In our inhibition 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, in order to calculate r_1 values. The binding parameters of the drug were estimated by non-linear least-squares simultaneous fitting of Eqs. (10) and (11) to the experimental data (r_1, F_1) using the MINSQ non-linear least-squares fitting program. The total drug concentration is known and constant while the free drug concentration F_2 was treated as an implicit variable and was calculated either algebraically or numerically by a

Table 2
Calculated $ADI_{1\%}$ values for the drugs studied[a]

Drug	$C_{D,1\%}$ ($\times 10^{-3}$ M)[b]	$ADI_{1\%}$ (M^{-1})
Ibuprofen	2.68	3.72
Naproxen	5.45	1.83
Ketoprofen	2.94	3.39
Indobufen	1.20	8.32
Sodium salicylate	4.65	2.14
Tolmetin	8.84	1.13
Phenylbutazone	1.20	8.32
Azapropazone	1.02	9.78
Iopanoic acid	2.10	4.75

[a] ANS concentration 2×10^{-4} M.
[b] Determined from the respective F_{ANS} vs. $C_{D(i)}$ plot.

built-in root finder of the program for each iteration 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 non-linear least-squares fitting of the Scatchard model to the experimental data of a 'blank' experiment, i.e. in the absence of drug.

Table 3 shows the results derived from the binding study of several drugs using the uncorrected as well as the corrected general competitive model. Both approaches resulted in quite similar estimates for the model parameters. This is probably attributed to the high binding affinity of these drugs for BSA; thus, very low free drug concentration values prevailed during the binding experiment, which did not interfere with the ANS electrode due to the rather low $K^{pot}_{ANS,D}$ values calculated (Table 1). The only statistically significant difference was found for phenylbutazone which exhibits a rather high $K^{pot}_{ANS,D}$ value (24×10^{-3}) (Table 3). On the other hand, the strong interfering effect of the two drugs with the highest $K^{pot}_{ANS,D}$, warfarin ($K^{pot}_{ANS,D} = 42\times10^{-3}$) and diflunisal ($K^{pot}_{ANS,D} = 116\times10^{-3}$) (Table 1), did not allow any valid binding experiment to be performed. In all cases the correlation coefficients of the fitting were extremely high for both models studied (Table 1).

The estimates for binding constants are in good agreement with reported values obtained using a variety of techniques (Vallner, 1977; Kober and Sjoholm, 1980; Kragh-Hansen, 1988; Li et al., 1988; Diana et al., 1989; Lapicque et al., 1993). For Naproxen, reported k_1 values range from 0.2×10^5 to 18.0×10^5 M^{-1}; for azapropazone the reported k_1 values are between 0.5×10^5 and 9.1×10^5 M^{-1}, while k_2 ranges from 1.0×10^4 to 7.8×10^4 M^{-1}; for phenylbutazone the range of k_1 values is 1.0×10^5 to 13.2×10^5 M^{-1}, while k_2 values have also been reported ranging from 0.6×10^4 to 0.74×10^4 M^{-1}; for salicylate, k_1 values range from 0.5×10^5 to 1.9×10^5 M^{-1} and k_2 is reported as 1.6×10^4 M^{-1}; for ketoprofen the k_1 value is reported to be 6.18×10^5 M^{-1}. Most of the calculated n_1 values are in agreement with previously reported values. However, rather high values of n_1, although not unrealistic (Weder and Bickel, 1970), were calculated for ketoprofen, naproxen and indobufen. When a ligand is bound to energetically identical sites of a protein, cooperative binding of this ligand can be induced by the presence of a second ligand (Kolb and Weber, 1975). Therefore, the calculated high n_1 values may be attributed to cooperative binding of these drugs to BSA induced by ANS affecting the binding capacity of the protein.

5. Conclusions

The displacement approach expands the usefulness of the potentiometric ion-probe technique to competitive protein binding studies. When interfering ionic drugs are encountered, $K^{pot}_{ANS,D}$ values can be estimated and used for correcting free ANS concentration readings. When moderate $K^{pot}_{ANS,D}$ values are found, these can be incorporated into the modified model of competitive binding and used for fitting purposes.

References

Angelakou, A., Valsami, G., Koupparis, M., Macheras, P., 1993. Use of 1-anilino-8-naphthalenesulphonate as an ion probe for the potentiometric study of the binding of sulfonamides to bovine serum albumin and plasma. J. Pharm. Pharmacol. 45, 434–438.

Angelakou, A., Sideris, E., Valsami, G., Koupparis, M., Macheras, P., 1994. 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. J. Pharm. Sci. 83, 1150–1154.

Bagattoli, L., Kivatiniz, S., Aguilar, F., Soto, M., Sotomayor, P., Fidelio, G., 1996. Two distinguishable fluorescent modes of 1-anilino-8-napthalenesulfonate bound to human albumin. J. Fluoresc. 6, 33–40.

Christopoulos, T., Diamandis, E., 1990. Binding studies using ion-selec-

Table 3
Estimated binding parameters of various NSADs using the general competitive uncorrected, Eqs. (6) and (7) (first row), and corrected, Eqs. (8) and (9) (second row), model[a]

Drug	n_1	$10^5\times k_1$ (M^{-1})	n_2	$10^4\times k_2$ (M^{-1})	R^2
Ketoprofen	8.8 (0.2)	10.8(2.0)			0.9990
$K^{pot} = 3.4\times10^{-3}$	8.8 (0.2)	10.5 (2.0)			0.9990
Naproxen	9.1 (0.3)	9.4 (2.2)			0.9993
$K^{pot} = 1.1\times10^{-3}$	9.1 (0.3)	9.3 (2.0)			0.9994
Phenylbutazone	3.2 (0.1)	1.4 (0.2)			0.9998
$K^{pot} = 24\times10^{-3}$	3.5 (0.1)	1.1 (0.2)			0.9998
Azapropazone	0.5 (0.7)	7.8 (2.6)	26.3 (3.2)	1.9 (0.4)	0.9999
$K^{pot} = 3.44\times10^{-3}$	0.5 (0.7)	7.9 (2.5)	26.4 (3.2)	1.8 (0.4)	0.9999
Indobufen	5.8 (2.2)	5.8 (3.4)	19.9 (1.4)	3.8 (1.4)	0.9997
$K^{pot} = 1.34\times10^{-3}$	5.8 (2.2)	5.7 (3.4)	19.9 (1.4)	3.8 (1.4)	0.9997
Salicylate	2.6 (1.1)	1.8 (0.6)	21.5 (3.1)	1.0 (0.2)	0.9999
$K^{pot} = 1.97\times10^{-3}$	2.8 (1.0)	1.8 (0.6)	21.5 (3.1)	1.0 (0.2)	0.9999

[a] Numbers in parentheses are withinrun standard deviations of the estimates. Temperature 25°C, pH 7.4. Uncorrected data taken from Angelakou et al. (1993).

tive electrodes. Examination of the picrate–albumin interaction as a model system. Anal. Chem. 62, 360–367.

Diana, F., Veronich, K., Kapoor, A., 1989. Binding of nonsteroidal anti-inflammatory agents and their effect on binding of racemic warfarin and its enantiomers to human albumin. J. Pharm. Sci. 78, 195–199.

Essassi, D., Zini, R., Tillement, J-P., 1990. Use of 1-anilino-8-naphthalenesulphonate as a fluorescent probe in the investigation of drug interactions with human alpha-1-acid glycoprotein and serum albumin. J. Pharm. Sci. 79, 9–13.

Kragh-Hansen, E., 1988. Evidence for a large and flexible region of human serum albumin possessing high affinity binding sites for salicylate, warfarin, and other ligands. Mol. Pharmacol. 34, 160–171.

Kober, A., Sjoholm, I., 1980. The binding sites on serum albumin for some nonsteroidal antiinflammatory drugs. Mol. Pharmacol. 18, 421–426.

Lapicque, F., Muller, N., Payan, E., Dubois, N., Netter, P., 1993. Protein binding and stereoselectivity of nonsteroidal anti-inflammatory drugs. Clin. Pharmacokinet. 25, 115–125.

Li, K., Thakur, A., Kapoor, A., 1988. Structural requirements for binding of nonsteroidal anti-inflammatory drugs to human serum albumin. J. Pharm. Sci. 77, 251–254.

Moody, G.J., Thomas, J.D.R., 1970. Ion Selective Electrodes, Merrow, Watford.

Nicolsky, B.P., 1937. Acta Phys. Chim., U.S.S.R. 7, 507.

Pacifici, G.M., Viani, A., 1992. Methods of determining plasma and tissue binding of drugs. Clin. Pharmacokinet. 23, 449–468.

Santos, E.C., Spector, A.A., 1974. Effects of fatty acids on the interaction of 1-anilino-8-naphthalene-sulfonate with human plasma albumin. Mol. Pharmacol. 10, 519.

Scatchard, G., 1949. The attraction of proteins for small molecules and ions. Ann. New York Acad. Sci. 51, 660–672.

Sideris, E., Valsami, G., Koupparis, M., Macheras, P., 1992. Determination of association constants in cyclodextrin/drug complexation using the Scatchard plot: application to β-cyclodextrin/anilino-naphthalene-sulphonates. Pharm. Res. 9, 1568–1574.

Sudlow, G., Birkett, D., Wade, D., 1975. The characterization of two specific drug binding sites on human serum albumin. Mol. Pharmacol. 11, 824–832.

Takisawa, N., Hall, D., Wyn-Jones, E., Brown, P., 1988. The construction and characteristics of drug-selective electrodes. Applications for the determination of complexation constants of inclusion complexes with α- and β-cyclodextrins including a kinetic study. J. Chem. Soc., Faraday Trans. 84, 3059–3070.

Vallner, J.J., 1977. Binding of drugs by albumin and plasma protein. J. Pharm. Sci. 66, 447–465.

Valsami, G., Macheras, P., Koupparis, M., 1990. Binding studies with cyclodextrins using ion-selective electrodes. J. Pharm. Sci. 79, 1087–1094.

Valsami, G., Macheras, P., Koupparis, M., 1991. Binding study of the fluorescent probe 1-anilino-8-naphthalene sulphonate to human plasma and human and bovine albumin using potentiometric titration. Pharm. Res. 8, 888–892.

Valsami, G., Koupparis, M., Macheras, P., 1992. Complexation studies of cyclodextrins with tricyclic antidepressants using ion-selective electrodes. Pharm. Res. 9, 94–100.

Watanabe, S., Saito, T., 1992. Conformational effects in the interaction of phenylbutazone with albumin studied by circular dichroism. Biochem. Pharmacol. 43, 931–935.

Wilding, G., Feldhoff, R.C., Vesell, E.S., 1977. Concentration-dependent effects of fatty acids on warfarin binding to albumin. Biochem. Pharmacol. 26, 1143.