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Molecular interactions between some non-steroidal anti-inflammatory drugs (NSAID's) and bovine (BSA) or human (HSA) serum albumin estimated by means of isothermal titration calorimetry (ITC) and frontal analysis capillary electrophoresis (FA/CE)

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ABSTRACT

The interactions between some non-steroidal anti-inflammatory drugs, NSAIDs, (naproxen, ibuprofen and flurbiprofen) and bovine (BSA) or human (HSA) serum albumin have been examined by means of two complementary techniques, isothermal titration calorimetry (ITC) and frontal analysis/capillary electrophoresis (FA/CE). It can be concluded that ITC is able to measure with high precision the strongest drug-albumin interactions but the higher order interactions can be better determined by means of FA/CE. Then, the combination of both techniques leads to a complete evaluation of the binding profiles between the selected NSAIDs and both kind of albumin proteins. When BSA is the binding protein, the NSAIDs show a strong primary interaction (binding constants: 1.5×10^7, 8×10^5 and 2×10^6 M^{-1} for naproxen, ibuprofen and flurbiprofen, respectively), and also lower affinity interactions of the same order for the three anti-inflammatories (about 1.7×10^4 M^{-1}). By contrast, when HSA is the binding protein two consecutive interactions can be observed by ITC for naproxen (9×10^5 and 7×10^4 M^{-1}) and flurbiprofen (5×10^6 and 6×10^4 M^{-1}) whereas only one is shown for ibuprofen (9×10^5 M^{-1}). Measurements by FA/CE show a single interaction for each drug being the ones of naproxen and flurbiprofen the same that those evaluated by ITC as the second interaction events.

Then, the ability of both techniques as suitable complementary tools to establish the whole interaction NSAIDs-albumin profile is experimentally demonstrated and allows foreseeing suitable strategies to establish the complete drug-protein binding profile. In addition, for the interactions analyzed by means of ITC, the thermodynamic signature is established and the relative contributions of the enthalpic and entropic terms discussed.

1. Introduction

It is well known that a drug present in the blood binds plasmatic proteins, such as any glycoprotein or albumin, in a variable degree and a rapid and reversible equilibrium is, very often, established between bound and free drug species. These proteins favor the solubility of the drugs and also act as drug carriers to the drug's specific target. Since only the free drug is able to interact efficiently with the target, to become of therapeutical interest, the interaction between the drug and the carrier should be strong enough to facilitate the transport but also weak enough to release the drug to the target. Thus, the quantitative study of the binding thermodynamics allows an improved knowledge of the drug pharmacokinetics [1–4].

In this study, bovine (BSA) and human (HSA) serum albumins are the selected proteins. It is well known that the albumin structure shows eight and half loops due to disulfur bridges and cystine residues. These loops can be grouped in three homologous domains and each one of them shows two big loops separated by another which is smaller. In fact, the serum albumins from all vertebrates show the same loop structure. However, despite the physico-chemical characteristics do not differ significantly, BSA and HSA are structurally similar just in about 76%. It was proposed that most of drugs bind the albumin through two main sites, known as Sudlow site I and Sudlow site II [5–7]. The non-steroidal anti-inflammatory drugs (NSAIDs) selected for this work are naproxen ($pK_a=4.38$), ibuprofen ($pK_a=4.54$) and flurbiprofen ($pK_a=4.24$) [8,9], being all of them α-aryl carboxylic acids. Then, at neutral pH the drugs are in their anionic form and they bind, preferably, the Sudlow site II of the albumin, as widely reported [5,6,10]. It is well known that these drugs interact with albumin but a variety of binding values were obtained when

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different experimental conditions and techniques are used for the measurements.

Among the techniques able to evaluate the interaction thermodynamics, only the isothermal titration calorimetry (ITC) can measure simultaneously the thermodynamic binding constant (K_b) closely related to free energy variation (ΔG), the enthalpy (ΔH) and entropy (ΔS) variations and also the interaction stoichiometry (n) [11]. Other commonly used techniques are able to measure the ratio between bound and free species concentrations and, then, to provide the stoichiometry and the binding constant. The enthalpy variation involved in the process could also be obtained by microcalorimetry and, in some other instances, from any indirect way. Thus, ITC seems to be the best experimental approach to get a complete description of the studied interaction. Tables 1 and 2 show the literature binding parameters and the used experimental approaches. It should be noticed that: a) only one interaction between naproxen and the BSA is reported, but two successive interactions were described for ibuprofen and flurbiprofen in specific conditions; b) despite several experimental approaches show only one drug-HSA interaction, two consecutive interactions have been also reported for the selected NSAIDs and HSA and c) in some instances, the binding constant values for a specific drug-albumin interaction determined by different authors and techniques but under similar experimental conditions (i.e. pH 7.0–7.4 achieved through phosphate buffer 50–100 mM, and 25–37 °C) are not consistent enough. In summary, the information shown in Tables 1 and 2 offers an illustrative overview about the wide variety of experimental approaches reported for the evaluation of the selected drug-albumin interactions. Thus, this evident experimental effort strongly supports their biological interest.

As shown in Tables 1 and 2, a variety of measurement techniques have been employed for K_b determination. In this work, ITC has been selected since, as explained, it measures directly the energy involved in the interaction and it is able to evaluate n, ΔH, ΔS and K_b (that is, ΔG) of any process through only one well designed titration. However, the obtained values are global measurements resulting from the contribution of the main interaction plus those of eventually associated side processes. Then, the resulting binding parameters strongly depend on the experimental conditions (for instance, pH, nature and concentration of the buffer, ionic strength and others) and, consequently, they should be considered conditional values, that is, values strongly dependent on the measurement conditions, which should be precisely described. When the thermodynamics of all the side reactions is well known, the true thermodynamic binding constant can be easily derived. Nevertheless, this computation is available only for reactions of low or intermediate complexity [35,36] but it is really difficult for interactions between a common drug and a complex protein such as the albumin. In these instances, it is not easy to identify precisely the side interactions although they can be, often, of significant relevance. Thus, it is a common practice to determine global binding constants in experimental conditions close to those of the biological environment where the interaction of interest occurs. In addition, it should be aware that ITC measurements are suitable when the binding constant, K_b, ranges between 10^8 and 10^4 [37,38] and, usually, this means that the technique is appropriate to measure the primary interactions but unable to evaluate properly the subsequent interactions, commonly weaker. For this reason the frontal analysis capillary electrophoresis (FA/CE), a technique able to measure higher order drug-protein interactions, has been selected too. Among the different CE techniques, FA/CE seems to be the most appropriate if the ratios between mobility values of the free drug, free protein and protein-drug entity are suitable. That is, the free drug mobility should differ enough of that of the protein-drug and the free protein and also these two last mobility values should be similar. These restrictions limit the number of systems to be studied but, for those that fulfill the mentioned conditions, FA/CE is a robust approach that allows the simultaneous determination of n and K_b [39–41]. Since this technique is able to determine K_b values between 10^5 and 10^2 [38], it allows the study of the higher order interactions. In some instances it could be possible independent measurements of the same interaction through both ITC and FA/CE approaches and to validate in this way the results obtained by the two methodologies.

Table 1
Literature data for interactions between some NAIDs and BSA.

n_1	K_{b1} (M^{-1})	ΔH (K cal/mol)	n_2	K_{b2} (M^{-1})	pH	T (°C)	Buffer (conc.)	Experimental technique*	Ref.
Naproxen									
0.76	8.33E+07	−14.00	–	–	7	10	Phosphate (10 mM)	ITC	[12]
0.82	4.75E+07	−15.80	–	–	7	15	Phosphate (10 mM)	ITC	[12]
0.77	3.06E+07	−17.60	–	–	7	20	Phosphate (10 mM)	ITC	[12]
0.61	3.68E+07	−23.10	–	–	7	25	Phosphate (10 mM)	ITC	[12]
0.58	1.80E+07	−24.80	–	–	7	30	Phosphate (10 mM)	ITC	[12]
0.57	1.14E+07	−28.90	–	–	7	35	Phosphate (10 mM)	ITC	[12]
0.90	2.90E+07	–	–	–	7.4	25	Phosphate (50 mM)	ITC	[13]
–	3.67E+07	−23.52	–	–	7.4	25	Phosphate (20 mM)	ITC	[14]
1.5	1.00E+07	–	–	–	7.4	25	Phosphate -	SIA	[15]
1.4	2.90E+06	–	–	–	7.4	39	Phosphate -	SIA	[15]
2.1	2.30E+05	–	–	–	7.4	51	Phosphate -	SIA	[15]
Ibuprofen									
–	3.20E+04	–	–	–	7.04	–	Phosphate (10 mM)	ACE	[16]
1.10	3.03E+05	–	–	–	7.4	25	Phosphate (50 mM)	ITC	[13]
–	2.53E+04	–	–	–	7.4	25	Phosphate -	FS[a]	[17]
–	3.20E+03	–	–	–	7.4	25	Phosphate -	FS[b]	[17]
1	2.25E+0.6	–	8	1.36E+04	7.4	25	Phosphate (67 mM)	ED	[18]
–	1.31E+06	–	–	1.76E+04	7.4	37	Phosphate -	ED	[19]
–	1,27E+06	–	–	8.02E+04	8.50	25	Borate -	ACE	[20]
Flurbiprofen			–						
–	2.80E+05	–	–	8.5E+03	7.04	–	Phosphate (10 mM)	ACE	[16]

*Acronyms, ITC: Isothermal titration calorimetry; SIA: Sequential Injection Affinity Chromatography; ACE: Affinity Capillary Electrophoresis; ED: Equilibrium Dialysis; FS: Fluorescence Spectroscopy.

[a] FS Competitive binding method;
[b] FS Non-competitive binding method.

Table 2
Literature data for interactions between some NSAIDs and HAS.

n_1	K_{b1} (M^{-1})	ΔH_1 (K cal/mol)	n_2	K_{b2} (M^{-1})	pH	T (°C)	Buffer (conc)	Experimental technique*	Reference
Naproxen									
–	1.43E+06	–	–	–	7.0	25	Phosphate (50 mM)	NMR	[21]
–	5.50E+05	–	–	–	7.4	25	Phosphate (100 mM)	CD	[22]
–	1.20+E06	–	–	1.40E+05	7.4	37	Phosphate (66 mM)	ED	[23]
–	9.00E+04	-6.50	–	–	7.4	25	Phosphate (100 mM)	MC	[24]
1.00	2.49E+04	–	–	–	7.4	37	Phosphate (100 mM)	ED	[25]
1.40	1.40E+06	–	–	–	7.4	–	Phosphate (50 mM)	ED	[26]
1.00	1.80E+06	–	–	–	7.4	–	Phosphate (50 mM)	Partition/HSA-Microparticles	[26]
–	3.90E+05	–	–	–	7.4	–	Phosphate (10 mM	FS	[27]
1	3.82E+06	–	5	6.89E+03	7.5	–	Tris (50 mM)	UF	[28]
–	8.00E+05	–	–	–	9.0	23	Borate (200 mM)	CE	[29]
Ibuprofen									
–	2.00E+06	–	–	–	7.0	25	Phosphate (50 mM)	NMR	[21]
–	2.40E+04	–	–	–	7.04	–	Phosphate (10 mM)	ACE	[16]
–	8.30E+03	–	–	–	7.4	25	Phosphate (50 mM)	FS[a]	[17]
–	2.40E+03	–	–	–	7.4	25	Phosphate (50 mM)	FS[b]	[17]
–	1.40 E+05	–	–	–	7.4	25	Phosphate (100 mM)	CD	[22]
–	1.60 E+05	-3.85	–	–	7.4	25	Phosphate (100 mM)	MC	[24]
0.98	4.35E+05	–	5.12	8.59E+03	7.4	37	Phosphate	ED	[30]
1.10	8.60E+04	–	–	–	7.4	37	Phosphate (100 mM)	ED	[25]
–	1.40E+05	–	–	3.6E+04	7.4	–	Phosphate (50 mM)	ΔCD	[31]
1.00	2.73E+06	–	6.27	1.95E+04	7.4	37	Phosphate (33 mM)	ED	[19]
1.00	1.76E+05	–	–	–	7.4	37	Phosphate	ED	[32]
1.10	1.20E+06	–	–	–	7.4	–	Phosphate (50 mM)	ED	[26]
1.00	1.30E+06	–	–	–	7.4	–	Phosphate (50 mM)	Partition/HSA-Microparticles	[26]
–	8.00E+04	–	–	3.5E+04	7.4	37	Phosphate (66 mM)	ED	[23]
1.0	7.1E+05	–	7.6	1.4E+04	7.4	37	Phosphate (67 mM)	CE/FA	[33]
1	3.56E+06	–	6	1.78E+04	7.4	37	Phosphate (67 mM)	ED	[18]
1.13	7.93E+05	–	9.67	2.22E+04	7.4	37	Phosphate (67 mM)	ED	[34]
–	2.97E+06	–	–	7.07E+04	8.50	25	Borate (60 mM)	ACE	[20]
–	1.00E+06	–	–	–	9.0	23	Borate (200 mM)	CE	[28]
Flurbiprofen									
–	1.30E+05	–	–	6.70E+03	7.04	–	Phosphate (10 mM)	ACE	[16]
0.97	8.62E+05	–	5.53	1.89E+04	7.4	37	Phosphate	ED	[30]
0.90	3.35E+04	–	–	–	7.4	37	Phosphate (100 mM)	ED	[25]
1.30	4.10E+06	–	–	–	7.4	–	Phosphate (50 mM)	ED	[26]
1.00	5.00E+06	–	–	–	7.4	–	Phosphate (50 mM)	Partition/HSA-Microparticles	[26]
–	1.20E+06	–	–	1.40E+05	7.4	37	Phosphate (66 mM)	ED	[23]
1.19	1.38E+06	–	7.69	8.98E+03	7.4	37	Phosphate (67 mM)	ED	[34]

*Acronyms. CE: Capillary Electrophoresis; NMR: Nuclear Magnetic Resonance; CD: Circular Dicroism; ΔCD: Difference Circular Dicroism; MC: Microcalorimetry; HPLC: High Performance Liquid Chromatography; ED: Equilibrium Dialysis; FS: Fluorescence Spectroscopy ; CE/FA: Capillary Electrophoresis/Frontal Analysis; ACE: Affinity Capillary Electrophoresis; UF: Ultrafiltration.

[a] FS Competitive binding method;
[b] FS Non-competitive binding method.

The main purpose of this study is the evaluation of ITC and FA/CE techniques as complementary tools to design a suitable strategy to face up the complete study of the drug-protein interactions. The selected biochemical systems deal on some well-known NSAIDs and two carrier proteins with close structure. These systems are partially known, simple enough and of outstanding interest as drug-protein interaction patterns. Therefore, they seem to be very suitable to go ahead with this study.

2. Experimental

2.1. Chemicals

Sigma-Aldrich bovine serum albumin (BSA) (> 99%) and human serum albumin (HSA) (99%) were used after purity verification by spectrophotometry [42]. Sigma-Aldrich naproxen (> 98%), ibuprofen (> 98%), flurbiprofen (> 97%), (N-2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (> 99.5 %) all of them used as received. NaOH 0.5 M (Titrisol, Merck) or HCl 0.5 M (Titrisol, Merck) were used to adjust the pH. Water purified by a Milli-Q-plus system with a resistance higher than 18 MΩ cm was used to prepare the buffer solutions and to clean the microcalorimeter.

HEPES buffer solution (pH 7.4 and ionic strength 50 mM) was prepared forming the sodium salt (Na^+HEPES^-) by adding NaOH to a 0.2 M HEPES solution, diluting it to get a 50 mM concentration and adding the required amount of HCl to achieve the desired pH. Working in this way the concentration of the buffer equals its ionic strength provided that it is calculated assuming that zwitterions do not contribute to the ionic strength of the solution [43].

2.2. ITC titrations

2.2.1. Instruments

The Microcal VP-ITC (MicroCal, LLC, Northampton, Ma, USA) titrator was used for the calorimetric titrations. The solutions were previously degassed by means of a vacuum degasser Thermovac (MicroCal, LLC, Northampton, Ma, USA). pH was measured with a Crison micro-pH 2002 potentiometer (Crison Instruments, Alella, Spain) equipped by a Crison 5014 combination electrode with a precision of ±0.1 mV (±0.002 pH units). The electrode system was standardized with ordinary aqueous buffers of pH 4.01 and 7.00.

2.2.2. Procedure

The albumin and drug solutions were prepared with the HEPES buffer. Both BSA and HSA solutions were 0.02 mM but the concentration of drug solutions ranged from 0.1 to 2.0 mM. All solutions were degassed for a period of 5 min at 24 °C prior their use. The titrations were performed at 25 ± 0.2 °C. The power reference was 10 μ cal s^{-1} and the stirring rate was 290 rpm to ensure rapid mixing. The injection volume was 8 μL and the interval between injections was 240 s to guaranty the equilibrium in each titration point. The syringe was filled with the drug solution whereas the albumin solution was in the cell. Background titrations consisting in identical titrant solution with the same cell filled just with the buffer solution and also successive buffer additions to the protein solutions were carried out to determine the background heat to be subtracted to the main experiment. Moreover, to evaluate the dilution heat of the drug-protein complex, successive buffer additions to the complex solutions were also performed. Each assay was repeated several times.

2.2.3. Calculations

The obtained data were analyzed through the Origin 7.0 software supplied by Microcal. The ITC data were collected automatically and analyzed to get n, ΔH, K_b, ΔG and ΔS associated with the interaction. In each instance the suitable adjusting model (one, two or sequential binding sites) should be introduced into the software.

2.3. CE/FA measurements

2.3.1. Instrument

A capillary electrophoresis Beckman P/ACE System 5500 (Palo Alto, CA, USA) equipped with a diode array detector operating at 214 nm was used. The measurements were carried out at 25 ± 0.1 °C on an uncoated fused-silica capillary (50 cm × 50 μm ID) obtained from Polymicro Technologies (Phoenix, AZ, USA). The working conditions included the application of a 15 kV voltage and positive polarity.

2.3.2. Procedure

Before first use, any new capillary was conditioned as follows: 10 min with water, 20 min with 1.0 M NaOH, 5 min with water, 10 min with 0.1 M NaOH, 5 min with water and, finally, 20 min with the running buffer. Before each working session, the capillary was rinsed 5 min with water, 10 min with 0.1 M NaOH, 5 min with water and 20 min with the running buffer. Finally, between runs the rinsing sequence was 1 min of water, 2 min of 0.1 M NaOH, and 3 min with the running buffer. At the end of the working session, the capillary was rinsed again with water for 10 min.

The measurements were performed by FA/CE and the HEPES buffer was used as the separation solution. Thus, solutions of free drug were prepared to build the calibration curve. Moreover, 20 solutions of a constant content of albumin (57 μM for BSA and 12 μM for HSA) and a variable concentration of drug solutions (naproxen, ibuprofen or flurbiprofen) in HEPES buffer were also prepared. Their concentration range depends on the studied drug. To obtain the "plateau" signal the sample was injected hydrodynamically at 0.5 psi. Various injection times in the 80–200 s range have been tested to look for the steady-state working conditions [44,45]. The injection volume was about 120 μL.

2.3.3. Calculations

The experimental data were exported from the software embodied in the Beckman P/ACE Station to an Excel sheet to record the "plateau" height, which is a measurement of the free drug concentration. Thus, the experimental data were treated according to :

$$r = \frac{[D_b]}{[P]_{total}} = \sum_{i=1}^{m} n_i \frac{K_{bi}[D_f]}{1 + K_{bi}[D_f]} \quad (1)$$

where $[D_b]$ and $[D_f]$ stand for the concentration of the bound drug and free drug, respectively, and $[P]_{total}$ for the total concentration of protein. n_i is the maximum number of the equivalent binding sites on the protein and K_{bi} the associated binding constant. Value of m denotes the total number of different binding sites that can be established by a particular drug-protein system and parameter r stands for the ratio between the concentrations of bound drug and total protein [38,44].

To calculate the interaction parameters, the binding model has been applied according to three different ways: a) by direct adjust of experimental points to Eq. (1) through the TableCurve 2D 5.01 software package, b) by the Scatchard linearization, Eq. (2), and c) by the Klotz linearization, Eq. (3) [38,44,46].

$$\frac{1}{r} = \frac{1}{nK_b[D_f]} + \frac{1}{n} \quad (2)$$

$$\frac{r}{[D_f]} = nK_b - rK_b \quad (3)$$

The two linearized models allow easily visualization of the successive interactions and the dispersion of the experimental data irrespective to the model. However, the best values are those obtained by the first approach.

3. Results and discussion

3.1. Interactions between NSAID's and BSA

3.1.1. ITC measurements

The experimental conditions to carry out the titrations have been chosen according the literature values shown in Table 1. Thus, the C parameter ($C = nK_b[P]_{total}$) should be between 5 and 500 and the titrant concentration high enough to generate a minimum heat of 3–5 cal per addition. Consequently, solutions about 0.02 mM of BSA and from 0.1 to 2.0 mM of drug have been used. This means that the naproxen titrations have been performed under optimal conditions and those for ibuprofen and flurbiprofen under a less suitable, but good enough, experimental conditions. Since the concentration of drug solution is a key parameter to define the titration curve, Fig. 1 shows two graphs for each NSAID as well as the corresponding blank curves for those titrations selected to be studied deeper. Naproxen-BSA curves show a regular shape with only one evident interaction jump (Fig. 1A and D). Curves for ibuprofen and flurbiprofen (Fig. 1B and C) show a noticeable negative slope at higher molar ratio values suggesting the presence of a second interaction or a significant dilution heat of the complex. This last hypothesis, however, has been discarded since successive additions of buffer to the complex solution revealed an irrelevant amount of involved heat. When the drug concentration solution is properly selected (Fig. 1D–F), the titration curves allow the calculation of the interaction parameters. The results are shown in Table 3 which shows that all the studied NSAIDs interact with BSA with the same stoichiometry and the strongest affinity is shown by naproxen. Our measurements are consistent with those reported and obtained under similar conditions [12,13] and denote a minimum effect of the buffer agent, phosphate in literature or HEPES in this work, despite their different chemical nature, concentration, and dissociation enthalpies ($\Delta H_{diss(H_2PO_4^-)} = 3.6$ kJ mol^{-1} and $\Delta H_{diss(HEPES)} = 20.4$ kJ mol^{-1}) [47]. This fact indicates that the binding reaction is not coupled with gain or release protons by the protein or the drug [48]. Therefore, the measured enthalpy variation should be very close to the true drug-protein binding enthalpy since no side

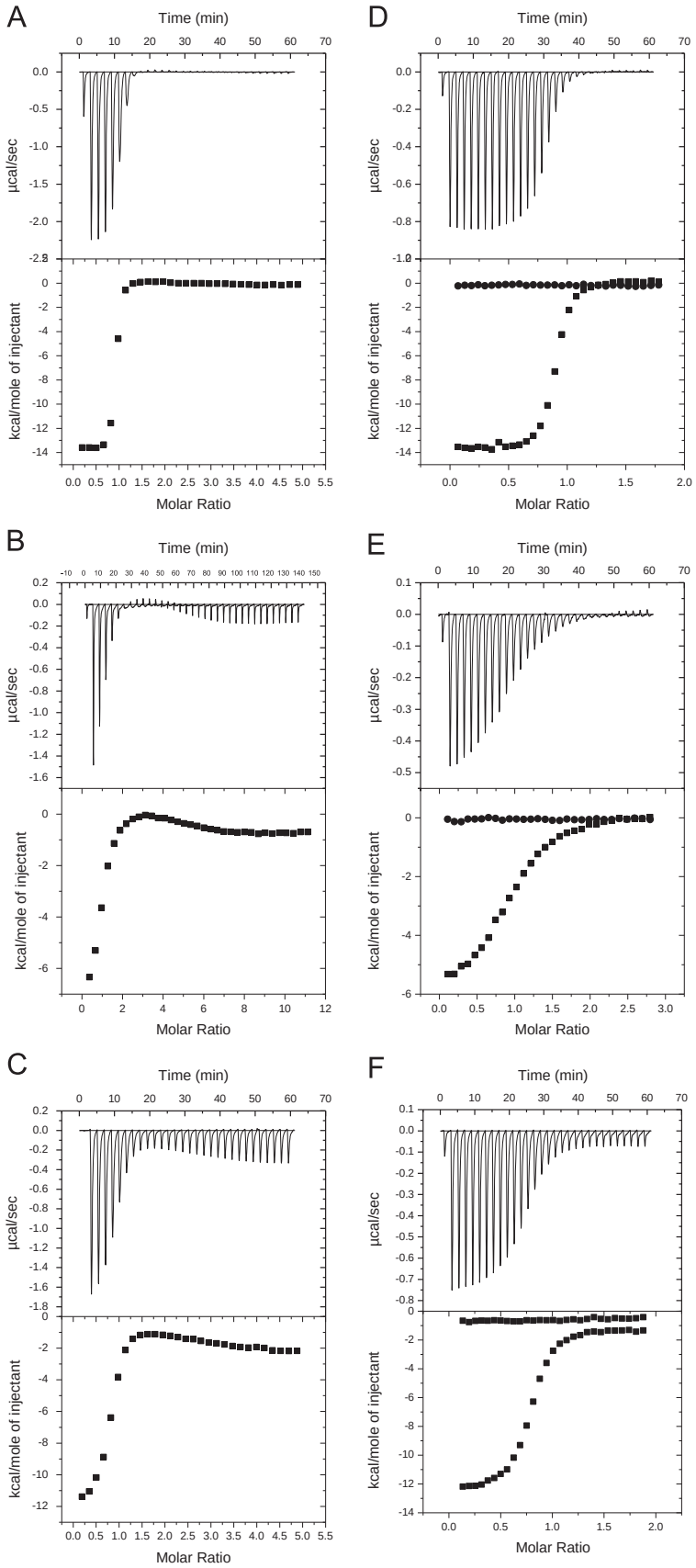

Fig. 1. BSA calorimetric titration curves along various molar ratio ranges. Titrant solutions: naproxen 0.54 mM (A) and 0.19 mM (D); ibuprofen 1.05 mM (B) and 0.31 mM (E); flurbiprofen 0.55 mM (C) and 0.21 mM (F).

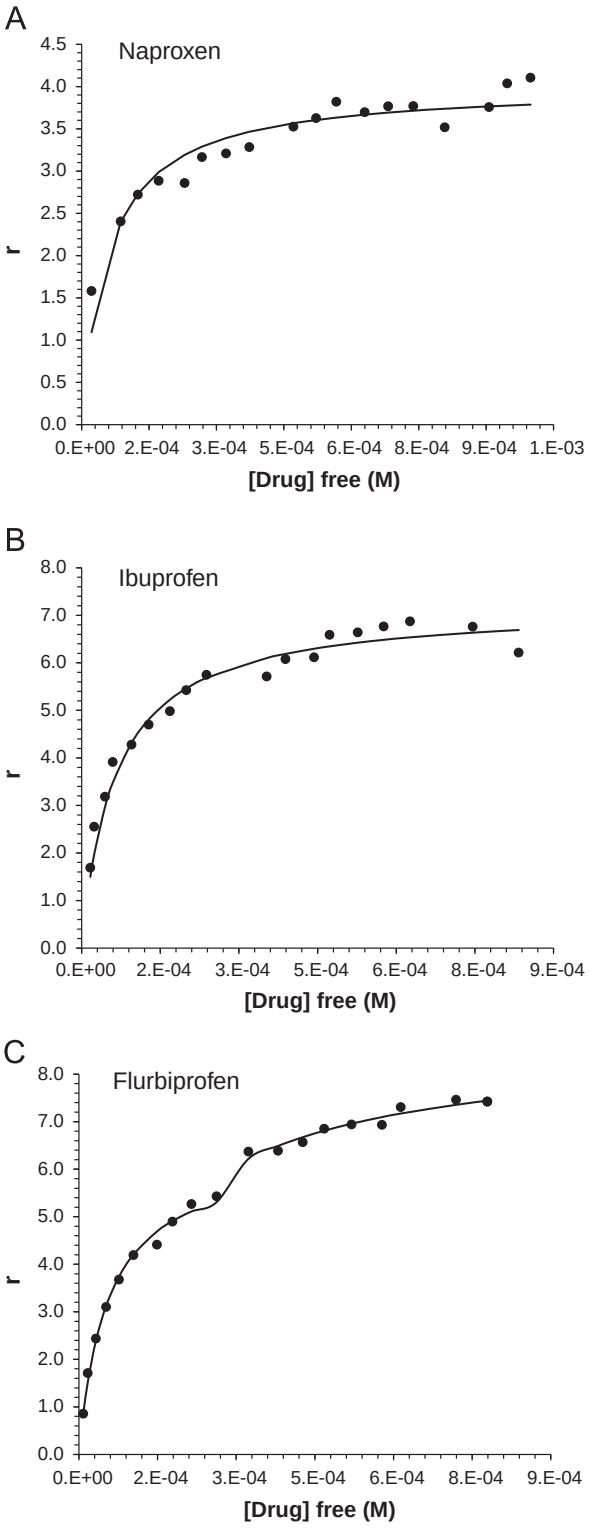

Fig. 2. Binding curves of NSAIDs–BSA interactions according to Eq. (1).

reactions in the reaction cell are foreseeable, as already suggested [12]. Moreover, according to data shown in Tables 1 and 3, the naproxen-BSA interaction seems to be less sensitive to the ionic strength than to the temperature. Nevertheless, measurements performed at $I=0.13$ M (50 mM phosphate) show that the interaction of ibuprofen-BSA is 100-fold weaker than for naproxen [13] whereas results achieved in this work at $I=0.05$ M (50 mM HEPES) point out a 19-fold times ratio. Then, ionic strength seems to be of some relevance in the final results.

Table 3 shows similar thermodynamic signatures of the main drug-BSA interaction for naproxen and flurbiprofen, denoting exothermic processes which involve significant heat amounts. These enthalpic contributions suggest a favorable number of hydrogen bond contacts or Van der Waals interactions between BSA and the drug [11,49] as well as the unfavorable entropy term ($T\Delta S$) could indicate any kind of conformational change in the involved species. The ibuprofen profile also denotes the enthalpy predominance but the small positive entropic term leads to a global interpretation as a favorable hydrogen bonding along with a modest hydrophobic contribution [11].

It should be pointed out that, according to the results shown in Table 3, the working conditions fulfilled the experimental ITC requirements for the three compounds examined and, in fact, the final values have been determined from optimal titration conditions.

3.1.2. FA/CE measurements

A calibration curve for each antiinflamatory drug has been established from calibration solutions containing a constant amount of albumin and variable amounts of drug. Thus, in right experimental conditions, the *plateau* height in the electropherogram is a measure of the free drug concentration, $[D_f]$, and sample solutions can be easily analyzed. Despite ITC measurements do not suggest any additional naproxen-BSA interaction, a higher molar ratio range has also been explored by CE/FA to compare the behavior of the three NSAIDs along the same drug-protein molar ratio range. Surprisingly, a second interaction has been clearly registered for naproxen. Since this interaction is recorded in the range previously studied by ITC (Fig. 1A), it should be concluded that it does not fulfill the minimum heat change required for each addition and it is invisible through calorimetric measurements. The Scatchard and Klotz plots show a unique second order interaction for naproxen and ibuprofen but two different interaction events for flurbiprofen. Fig. 3 shows the experimental values for the analyzed drugs fitted to Eq. (1). Results are shown in Table 4. In all instances the second interaction is clearly differentiate from the first one determined by ITC (Table 3) and involves lower K_b values. Interestingly, Table 1 shows that some K_{b1} values given for ibuprofen as the only binding constant [16,17] agree with those reported for K_{b2} [18,19] or determined in this work (K_{b2},Table 4) and all these data should be attributed to the same drug-BSA interaction.

3.2. Interactions between NSAID's and HSA

3.2.1. ITC measurements

Interactions between NSAIDs and HSA have been evaluated in the way explained in Section 3.1.1. The shapes of the obtained ITC

Table 3
NSAIDs–BSA interaction parameters obtained by ITC.

	n_1	K_{b1} (M^{-1})	ΔH_1 (K cal/mol)	$T\Delta S_1$ (K cal/mol)	ΔG_1 (K cal/mol)	Molar ratio range	N
Naproxen	0.81 ± 0.06	$(1.5\pm0.8)\cdot10^7$	-14 ± 1	-4.3 ± 0.9	-9.7 ± 0.4	0–5	4
Ibuprofen	0.8 ± 0.2	$(8\pm3)\cdot10^5$	-6.5 ± 0.7	1.5 ± 0.8	-8.0 ± 0.2	0–3.5	6
Flurbiprofen	0.8 ± 0.1	$(2\pm1)\cdot10^6$	-12.1 ± 0.8	-4 ± 1	-8.5 ± 0.4	0–2.5	6

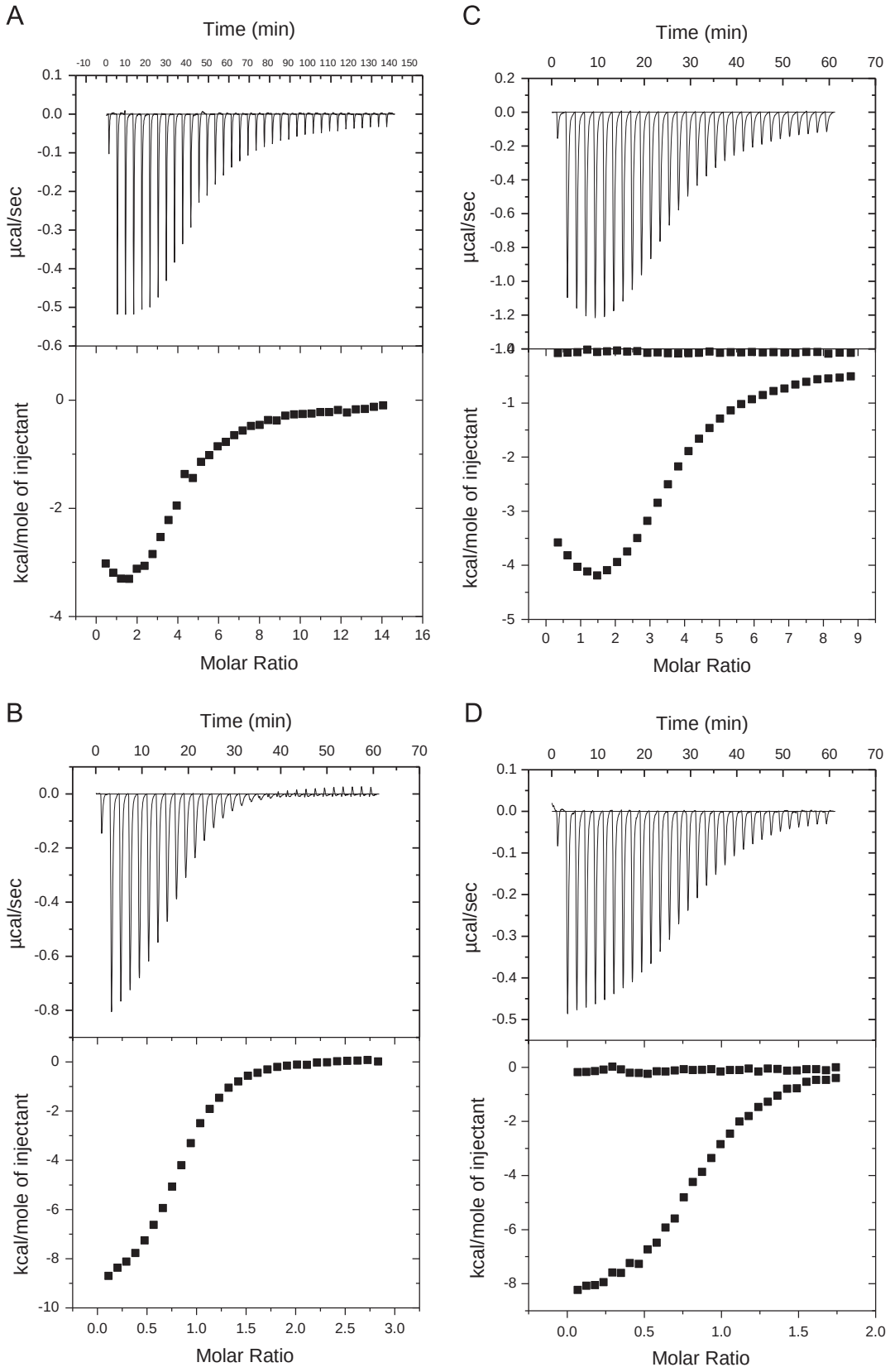

Fig. 3. HSA calorimetric titration curves along various molar ratio ranges. Titrant solutions: naproxen 1.31 mM(A) and 0.99 mM (C); ibuprofen 0.31 mM (B) and 0.20 mM (D).

curves differ significantly of those for NSAIDs–BSA, as shown in Figs. 3 and 4. Thus, two consecutive interactions are clearly recorded by the naproxen curve whereas ibuprofen generates a single jump. The fitting parameters are shown in Table 5. It should be noticed that no previous ITC studies were published about NSAIDs–HSA interactions and only two references describing two successive interactions for naproxen are reported [23,28] (Table 2). The obtained parameters for the strongest naproxen-HSA interaction

agree with those previously determined by various methods [21–23,26,28,29], whereas K_{b2} provides an intermediate value between the published ones [23,28]. However, values determined by microcalorimetry [24] are consistent with those achieved in this work for the second interaction and, probably, refer to the same process. Two consecutive interactions determined by a variety of techniques have been reported for ibuprofen showing a spread set of K_{b1} values but a nice consistency in K_{b2}, as shown in Table 2. The K_{b1} value determined in this work agrees with most previously published [18–21,26,28,31,34], (Table 5).

The significant enthalpy gap between the end of the jump of the flurbiprofen titration curve and the blank signal points out the presence of a second interaction, see Fig. 4A. Then, new titrations were performed using more concentrated drug solutions, Fig. 4B.

Table 4
NSAIDs–BSA interaction parameters obtained by CE/FA.

	n_2	K_{b2} (M^{-1})	n_3	K_{b3} (M^{-1})	s	Molar ratio range
Naproxen	4.0 ± 0.1	$(1.7 \pm 0.3) \cdot 10^4$	–	–	0.21	1 – 20
Ibuprofen	7.2 ± 0.2	$(1.6 \pm 0.2) \cdot 10^4$	–	–	0.40	1–20
Flurbiprofen	6.4 ± 0.2	$(1.8 \pm 0.1) \cdot 10^4$	8.6 ± 0.2	$(8 \pm 1) \cdot 10^3$	0.13	1–20

After subtracting the residual heat, both interaction events were evaluated separately and results shown in Table 5. For the first interaction results agree with most literature values [23,26,34], whereas parameters for the second one are not consistent with those previously published.

Table 5 shows that all interactions are exothermic being the most significant entropic term the one associated to the strongest interaction of naproxen. Then, hydrogen bonding and/or Van der Waals interactions as well as the hydrophobic effects, mainly related to the entropic contribution, favor naproxen-HSA interaction. The entropic term in the remaining instances is very low. Thus, except for the first naproxen-HSA interaction, all the analyzed processes seem to be dominated by the redistribution of the hydrogen bond network between the reacting species, including the solvent, and/or the Van der Waals interactions [11,49].

3.2.2. FA/CE measurements

NSAIDs–HSA interactions have been studied in the way already explained in Section 3.1.2 and the obtained data fitted to Eq. (1) and plotted in Fig. 5. Naproxen shows the worst fitting and the experimental points become erratic at drug-protein molar ratio higher than 15. Table 6 shows the calculated K_b value which agrees

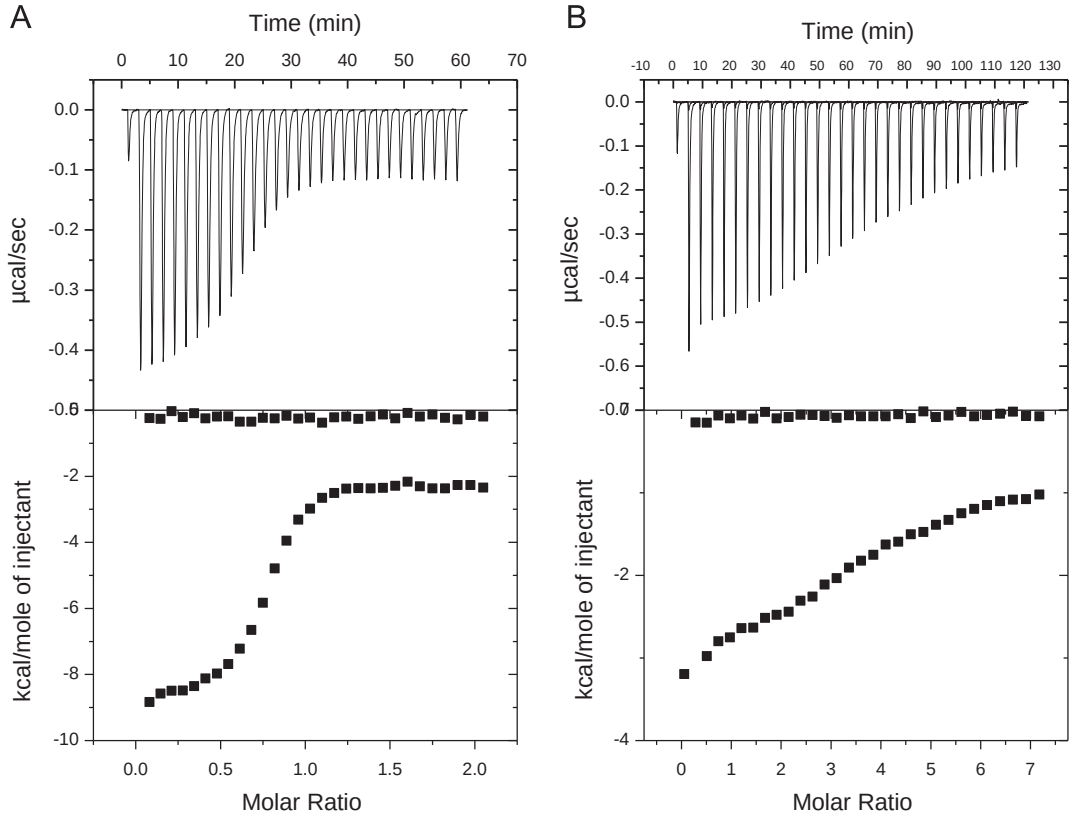

Fig. 4. HSA calorimetric titration curves along two molar ratio ranges. Titrant solution: flurbiprofen 0.23 mM (A) and 0.80 mM (B).

Table 5
NSAIDs–HSA interaction parameters obtained by ITC.

	n_1	K_{b1} (M^{-1})	ΔH_1 (K cal/mol)	$T\Delta S_1$ (K cal/mol)	ΔG_1 (K cal/mol)	n_2	K_{b2} (M^{-1})	ΔH_2 (K cal/mol)	$T\Delta S_2$ (K cal/mol)	ΔG_2 (K cal/mol)	Molar ratio range	N
Naproxen	1.0 ± 0.2	$(9 \pm 2) \cdot 10^5$	-3.1 ± 0.3	4.9 ± 0.4	-8.1 ± 0.2	2.5 ± 0.3	$(7 \pm 1) \cdot 10^4$	-6.4 ± 0.7	0.2 ± 0.8	-6.6 ± 0.1	0–9	9
Ibuprofen	0.84 ± 0.02	$(9 \pm 1) \cdot 10^5$	-8.9 ± 0.1	-0.8 ± 0.1	-8.10 ± 0.08	–	–	–	–	–	0–3	4
Flurbiprofen	0.71 ± 0.05	$(5 \pm 3) \cdot 10^6$	-10.3 ± 2	-1.3 ± 2	-8.9 ± 0.6	–	–	–	–	–	0–2.5	6
	–	–	–	–	–	4.8 ± 0.3	$(6 \pm 4) \cdot 10^4$	-4.55 ± 0.8	2 ± 1	-6.5 ± 0.4	1.5–8	4

with the one determined by ITC for the second interaction (Table 5) pointing out that it can be determined by both techniques despite the slight discrepancy in the estimated stoichiometry. Ibuprofen and flurbiprofen experimental points fit well the applied model until a molar ratio of 30 and 20, respectively. After these upper limits, systematic deviations showing opposite slope are observed denoting that new weaker interactions are still possible, at least for flurbiprofen. Obtained parameters for flurbiprofen agree with those determined by ITC for the second interaction showing again the ability of both techniques to catch the same interaction when it involves suitable stoichiometry and binding constant. Results obtained in this study (Tables 5 and 6) cannot be directly compared with those from the literature since most of them were obtained at physiological temperature, 37 °C, as shown in Table 2.

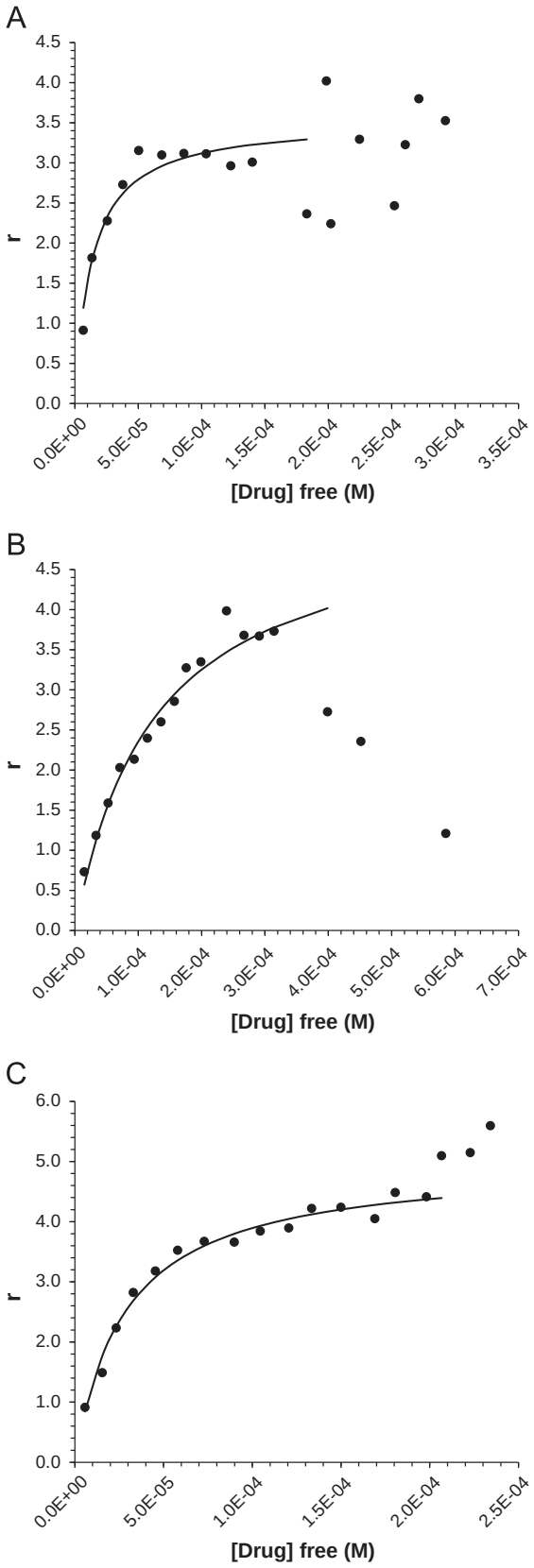

Fig. 5. Binding curves of NSAIDs–HSA according to Eq. (1).

3.3. Remark about NSAIDs-albumin interactions

According to Tables 1 and 2, a stoichiometry around 1 is commonly attributed to the first NSAID's-albumin interactions. This fact is consistent with the assessment that the anionic species of NSAIDs interact with albumin in the well-known Sudlow site II [5,6,10,50]. However, only few n_2 values are reported despite the used techniques are able to measure it. Probably, this is because of the lack of consistency between the results achieved from measurements performed under slightly different conditions. In fact, the achieved n_2 value results from the contribution of several unspecific interactions established by means of binding sites of similar energy and measured as a whole [38,44]. Then, the n_2 value estimated in a particular measurement is just a mean value which depends on the number of available binding sites, conditioned to some extend by the experimental conditions. This is the main reason of the lack of robustness of published n_2 values.

In order to verify this assessment, the naproxen-BSA system was also examined by CE/FA in a shorter range of concentration ratios, from 1.3 to 8.5, keeping all the remaining experimental conditions as described in Section 2.3. The final results show a consistent K_b value, $(2.3 \pm 0.4) \times 10^4$ M^{-1}, but the n_2 value of 2.5 which differs of the one shown in Table 4. Similarly, Tables 5 and 6 show some inconsistency in estimated n_2 values for naproxen-HSA interactions although very similar n_2 values are achieved for flurbiprofen. These results support the assumption of the unspecificity of the analyzed interactions and the convenience to consider the stoichiometry assignations for the higher order interactions just as a useful approximation.

3.4. Conclusions

The complete interaction profiles between anionic NSAIDs and BSA deal on a main interaction and one or two higher order interactions with energetic significance. The first interaction can be characterized from a thermodynamic point of view by ITC. The significant enthalpic contribution suggests a favorable number of hydrogen bond contacts or Van der Waals interactions between BSA and naproxen or flurbiprofen. In case of ibuprofen, however, the lower enthalpic heat together with the favorable entropic term indicates that the effect of hydrogen bonding is reinforced by a small hydrophobic contribution. The higher order interactions were successfully determined by CE/FA and the results reveal very similar binding values for the secondary interactions of all studied

Table 6
NSAIDs–HSA interaction parameters obtained by CE/FA.

	n_2	K_{b2} (M^{-1})	s	Molar ratio range[a]
Naproxen	3.5 ± 0.1	$(7.4 \pm 1) \cdot 10^4$	0.17	1.5–15
Ibuprofen	5.2 ± 0.2	$(8.1 \pm 0.8) \cdot 10^4$	0.12	2.0–30
Flurbiprofen	5.0 ± 0.1	$(3.5 \pm 0.3) \cdot 10^4$	0.14	1.5–20

[a] The upper limit refers to the fitted last point in Fig. 5.

compounds and also an additional weaker interaction for flurbiprofen. The higher order interactions show strength enough to be taken into account in the whole evaluation of the energetics of NSAID's interactions with BSA.

The parallel study carried out with HSA allows the establishment of the thermodynamic signatures of the two consecutive interactions for naproxen and for flurbiprofen as well as for the first ibuprofen-HSA interaction. Results reveal that naproxen interactions are due to hydrogen bonding and/or Van der Waals interactions and the first one is significantly favored by hydrophobic effects. Other analyzed interactions are, mainly, originated by the contribution of the enthalpic heat. The CE/FA analysis confirms the binding values for the second interaction of naproxen and flurbiprofen and shows a new one for ibuprofen. Even in this instance, the secondary interactions should be integrated in the energetic interaction profiles of NSAID's with HSA.

It should be noticed that first NSAIDs–BSA constants differ significantly between them whereas those of NSAIDs–HSA are of the same order of magnitude. By contrast, K_{b2} values are very similar in both instances. Then, the differences between molecular structure of BSA and HSA strongly affects the first interaction with NSAIDs in the Sudlow II site according to its specific character whereas the high order interactions show similar strength and unspecific character.

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