Binding Thermodynamics of Diclofenac and Naproxen with Human and Bovine Serum Albumins: A Calorimetric and Spectroscopic Study

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Abstract

Serum albumins are ubiquitous proteins able to bind a variety of exogenous and endogenous ligands including hydrophobic pharmaceuticals. Most drugs bind to two very active binding regions located within sub-domains IIA and IIIA of the protein, also known as Sudlow's sites. The drug binding mode of serum albumin provides important pharmacological information and influences drug solubility, efficacy, biological distribution, and excretion. Here, the binding thermodynamics of Diclofenac and Naproxen, two non-steroidal anti-inflammatory drugs (NSAIDs) to bovine and human serum albumins (BSA and HSA, respectively) were studied by isothermal titration calorimetry (ITC), fluorescence spectroscopy and differential scanning calorimetry (DSC). The ITC data show that the binding affinity (K) of Diclofenac to BSA and HSA is on the order of 10^4 M^{-1} with a binding stoichiometry (n) of 2 drug molecules per protein. Naproxen binding to the two proteins exhibits a different profile with K and n values on the order of 10^6 M^{-1} and 0.75 for BSA and 10^5 M^{-1} and 3 for HSA, respectively. The binding of the two drugs to HSA is found to be both enthalpically and entropically favored suggesting the formation of hydrogen bonds and van der Waals hydrophobic effects. Binding of the two drugs to BSA is only enthalpically favored with an unfavorable entropy term. Significant enthalpy–entropy compensation phenomena were reported for Diclofenac and Naproxen binding to BSA but not to HSA. Fluorescence quenching data between Diclofenac and the two proteins suggest static collisions and the formation of ground-state protein-drug complexes. The DSC data corroborate the ITC findings and show multiple sequential unfolding events and a strong drug stabilization effect at high drug to protein ratios. Overall, the calorimetric and spectroscopic data provided insights into the nature of these protein-drugs interactions and might offer useful information in future drug discovery studies.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are powerful analgesic, antipyretic, and anti-inflammatory agents that are among the most commonly prescribed medications worldwide [1]. Unlike steroidal drugs, NSAIDs are non-narcotics and operate by essentially blocking the COX enzymes with relatively minor side effects [2, 3]. While common NSAIDs are derivatives of organic acids such as salicylic, propionic, acetic, enolic, and anthranilic, other NSAID categories consist of sulfonanilides, derivatives of popular antibiotic sulfa drugs. For instance, Naproxen (trade names Aleve, Anaprox, Apranax, Naprelan, Naprosyn) belongs to the phenylacetic acid class of NSAIDs (2-(6-methoxy-2-naphthalen)propanoic acid), Diclofenac (trade names Aclonac, Cataflam, Voltaren) is a propionic acid derivative inferred from the chemical name 2-(2,6-dichloranilino) **phenylacetic acid**, and Celecoxib (trade names Celebrex and Celebra) is a sulfonamide-based type drug (4-[5-(4-Methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide) which in 2015 received strong warnings from the FDA and the American Heart Association that included an increased risk of major cardiovascular problems. Interestingly, the S-enantiomer of Naproxen is 28-fold more active as an anti-inflammatory drug than the R-isomer which is reported to be a liver toxin and to cause gastrointestinal disorders [4, 5]. The differences in the chemical structures and half-lives of these drugs and their binding to serum albumin influence several important pharmacological properties such as efficacy, solubility, bioavailability, toxicity, dosage, distribution, and excretion. In the circulatory system, both Diclofenac and Naproxen bind to a large extent to serum albumin (> 99%) with minimal accumulation in tissues. However, Diclofenac's half-life is at least 6 times shorter than Naproxen and is thus excreted much faster [6].

Mammalian serum albumin is the most abundant protein of the circulatory system (40–50 g/L). It plays an important role in the regulation of osmotic blood pressure, blood pH, and the transport of numerous endogenous and exogenous compounds such as fatty acids, amino acids, hormones, ions, metals, drugs and their byproducts [7]. It is a 67 kDa globular monomeric, hydrophilic, and non-glycosylated protein. It is arranged in a globular heart-shaped conformation and is composed of three α -helical domains (I, II and III), each with two subdomains (IA, IB, IIA, IIB, IIIA, IIIB) and several folded disulfide bridged loops with up to five different potential binding sites [8-11]. Two so-called Sudlow sites (site I and site II) are major binding regions within

subdomains IIA and IIIA that allow the protein to bind a variety of hydrophobic, heterocyclic and anionic compounds including 70% of man-made drugs [12-17]. Due to its versatile binding and transport properties, serum albumins are one of the best studied protein models that are well known for their involvement in the reversible binding and transport of free drugs in the plasma [8]. Because NSAIDs are normally insoluble in blood plasma, the two Sudlow sites have been proposed to decrease drug toxicity and increase drug solubility and transport [17]. Sudlow's site I has been shown to bind mostly bulky heterocyclic compounds such as coumarin compounds, sulfonamides, and salicylates while Sudlow's site II binds aromatic carboxylic acids and profens such as ibuprofen and ketoprofen [15-18]. The distribution, metabolism, and free concentration of administered drugs in the blood stream is strongly affected by the nature of protein-drug interactions, which can influence drug stability and toxicity. Therefore, the quantitative characterization of serum albumin-drug binding thermodynamics is essential to improving our knowledge of biomolecular recognition and drug pharmacokinetics. Human serum albumin (HSA) has been shown to protect the drugs it binds to from oxidative damage as well as change their pharmacokinetic and pharmacodynamic properties [7, 9, 19-22].

Two non-steroidal anti-inflammatory drugs, Diclofenac and Naproxen (Fig. 1) were chosen for this study owing to their wide use as over-the-counter pain and anti-inflammatory medications. Diclofenac has pKa values of 3.80-4.19 while Naproxen's pKa ranges between 4.26 - 4.76 [23]. At neutral pH, the two drugs are in their anionic forms and bind preferably to Sudlow site II of serum albumin as previously described [10, 16, 24]. Despite their high sequence and structural similarities (76%), the distribution and binding ability of serum albumin domains is not the same as evidenced in the different binding thermodynamics of several non-steroidal anti-inflammatory drugs with bovine (BSA) and human serum albumins [10 and references therein]. Whereas some agreement regarding the first strong NSAID-albumin binding site is reported in the literature, many inconsistencies exist for the second set of weaker, non-specific binding sites with stoichiometric ratios upward of 6 drug molecules per protein and a wide range of low and high affinity constants spanning five orders of magnitude [11 and refs therein]. These discrepancies are likely due to different experimental conditions and investigative techniques used to carry out the binding interactions.

Figure 1: Molecular structures of Diclofenac (-2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid sodium salt (left) and Naproxen (-6-Methoxy- α -methyl-2-naphthaleneacetic) acid sodium salt S-enantiomer.

The purpose of this study is to evaluate the interaction of Diclofenac and Naproxen with BSA and HSA using isothermal titration calorimetry (ITC), fluorescence spectroscopy, and differential scanning calorimetry (DSC). ITC and fluorescence spectroscopy are amongst the most commonly used techniques to evaluate the thermodynamics of biomolecular interactions and in particular the binding of NSAIDs to serum albumins. All three techniques employed here will allow a more in-depth characterization of the mechanism underlying drugs-serum protein interactions. As a universal calorimetric tool, ITC is able to provide in a single experiment all of the interaction thermodynamics including the binding affinity (K_a), the stoichiometry of binding (n), the enthalpy change (ΔH), the entropy change (ΔS), and the free energy change (ΔG) of the reaction allowing a complete thermodynamic description of the molecular interaction to be generated. The partitioning of the Gibbs free energy change into enthalpic and entropic contributions provides new insights into structure function relationships and a deeper understanding of the nature of these interactions and is particularly useful in drug discovery studies. Fluorescence spectroscopy relies on the quenching of the intrinsic fluorescence spectra of the protein following drug binding and allows the characterization of the type of binding interaction (static *vs.* dynamic) between the protein and the drug molecules. DSC can complement these measurements and assist in characterizing protein folding dynamics and determining the strength and stability of the protein-drug complexes by evaluating the temperature of denaturation (T_m), the heat capacity change (ΔC_p) and the enthalpy of thermal denaturation (ΔH).

Materials and Methods

Human serum albumin (HSA, $\geq 99\%$ purity, essentially globulin free), bovine serum albumin (BSA, $\geq 98\%$ purity, essentially protease free), high purity Diclofenac sodium salt and S-Naproxen sodium salt, were purchased from Sigma-Aldrich chemical company (St. Louis, USA) as lyophilized powders and used without further purification. Purified HSA and BSA were isolated from blood plasma and thus contain fatty acids. The two protein solutions were freshly prepared in 50 mM phosphate buffer, 50 mM NaCl, pH 7.40 using DI water and their concentrations checked at 280 nm using a Varian Cary 50-Bio UV-Vis spectrometer and a molar absorptivity (ϵ) value of $42,684 \text{ M}^{-1}\text{cm}^{-1}$ for HSA and $43,824 \text{ M}^{-1}\text{cm}^{-1}$ for BSA, respectively. Dibasic anhydrous sodium phosphate (Na_2HPO_4) was purchased from Fisher Scientific.

Specification of Chemical Samples

Chemical Name	Source	Initial Mole Fraction Purity	Purification Method	Final Mole Fraction Purity	Analysis Method*
Human serum albumin (HSA) – Prod# A8763; Lot# SLBK6136V	Sigma-Aldrich	≥ 0.99	Agarose Electrophoresis	1	Agarose Electrophoresis
Bovine serum albumin (BSA) – Prod# A4737; Lot# SLBD1578V	Sigma-Aldrich	≥ 0.98	Agarose Electrophoresis	1	Agarose Electrophoresis
Diclofenac sodium salt – Prod# D6899; Lot# BCBK6371V	Sigma-Aldrich (Originator: Novartis) Chemical synthesis	≥ 0.98	---	1	Thin-layer chromatography (TLC)
S-Naproxen sodium salt – Prod# M1275; Lot# SLBF9269V	Sigma-Aldrich (Originator: Bayer) Chemical synthesis	≥ 0.98	---	1	Titration with HClO_4
Dibasic anhydrous sodium phosphate – Prod# S3741	Fisher Scientific	---	Certified ACS Grade	≥ 0.99	Titration with 1M HCl

* The certificate of analysis is available on the company's websites.

Isothermal Titration Calorimetry

Isothermal titration calorimetry experiments were carried out on a TA Instruments low volume Nano ITC calorimeter with an active cell volume of 185 μL . All titrations were performed in 50 mM phosphate buffer, 50 mM NaCl, pH 7.40 with a stirring rate of 250 rpm using a 50 μL titrating syringe at temperatures ranging between 20 and 40 $^{\circ}\text{C}$. Typically, an automated sequence of 16 injections, each of 3 μL drug titrant into the sample cell containing serum

albumin, spaced at 5 min intervals to allow complete equilibration were performed with the equivalence point coming at the area midpoint of the titration. ITC data were collected automatically and analyzed using the NanoAnalyze software from TA Instruments and a mathematical model involving one class of independent binding sites. All ITC experiments were repeated at least three times to ensure reproducibility with background heat corrections from drug solution titrations into buffer alone to account for the heat of dilution/mixing. Conditions for each experiment are given in the figure captions and the reported errors are standard deviation (i.e. 0.68 confidence interval) from curve fitting and replicate measurements.

Differential Scanning Calorimetry

DSC experiments were performed on a Nano DSC from TA Instruments under a constant pressure of 3 atm in the temperature range of 25–100 °C using a scan rate of 1 °C/min. All samples (1 mg/ml) were prepared in 50 mM phosphate buffer, 50 mM NaCl pH 7.40 and degassed for 20 minutes prior to analysis using the TA Instrument degassing station. Protein-drug complexes were prepared in the same manner at different protein to drug ratios as indicated in the DSC caption of figure 6. The DSC raw data were corrected by subtracting a buffer – buffer background scan and analyzed using the TA Instruments DSC NanoAnalyze software. Model-independent calorimetric enthalpy (ΔH_{cal}) values were obtained from the area under the unfolding curves and the reported melting temperature (T_m) values are those at maximum heat capacity.

Fluorescence Spectroscopy

Fluorescence quenching measurements were performed on a Varian Cary Eclipse fluorimeter equipped with a QNW Peltier temperature controller. The experiments were conducted at 25.00 ± 0.01 °C in 50 mM phosphate buffer, 50 mM NaCl, pH 7.40 using 280 nm excitation wavelength. The bandwidth of the excitation and emission monochromators was set at 5 nm each and the fluorescence spectra were recorded in the range of 250–500 nm. Several injections of 2 μ L each of a drug solution at 625 μ M were made to a 10 μ M protein solution in 50 mM phosphate buffer, 50 mM NaCl, pH 7.40. The fluorescence quenching data were analyzed using OriginLab software version 8.

RESULTS AND DISCUSSION

ITC of Diclofenac and Naproxen Binding to BSA

To characterize the binding energetics of Diclofenac and Naproxen interaction with BSA, several ITC experiments were performed. We note that all ITC parameters reported here are apparent values (i.e. experimentally measured values under non-standard conditions) which may include possible contributions to the overall equilibrium from different processes such as structural rearrangements of drug-protein complexes upon interaction and drugs and buffer species in different states of protonation). However, given the small ionization enthalpy for phosphate buffer ($\Delta H_{\text{ionization, H}_2\text{PO}_4} = -3.6 \text{ kJ/mol}$), previous measurements under conditions similar to ours suggested that the binding reaction does not involve protons gain or loss by the protein or the drug upon binding and is essentially very close to the true drug-protein binding enthalpy [10, 12, 25, 26]. The top panels of Fig. 2 illustrate raw ITC results for Diclofenac and Naproxen titrations with BSA. The integrated heats ($\mu\text{J/s}$) for each injection vs. the molar ratio of drug to protein are shown in the insets of panels A and B after subtraction of the control heats due to dilution and mixing. The ITC upward positive peaks observed in Fig. 2 correspond to an exothermic reaction for Diclofenac and Naproxen binding to BSA. The binding experiments were conducted in the temperature range of 20–40 °C and all experimental thermodynamic parameters obtained from curve fitting of the integrated heats are compiled in Table 1. The data show one class of binding sites with a stoichiometry of two drug molecules per protein and dissociation constants K_d on the order of 20-50 μM at the studied temperatures. Overall, the binding is enthalpically favored and consistent with an enthalpy–entropy compensation phenomenon (more below). The unfavorable entropy change and favorable enthalpy change increase with increasing temperature (Table 1) suggesting that the interaction is mostly electrostatic in nature and consistent with the negatively charged Diclofenac molecule at neutral pH ($\text{pK}_a \sim 4$). However, the presence of two aromatic rings in Diclofenac might confer a hydrophobic character to this interaction whereby the aromatic moieties interact with Sudlow’s site II of BSA and the carboxylate group moiety with charged amino-acid residues in or around the hydrophobic cavity as reported in several crystallographic studies [11, 24, 27]. Our ITC data is also consistent with earlier studies suggesting that Diclofenac can interact with the protein via ionic and hydrophobic interactions [28-31].

Naproxen, on the other, binds to BSA more strongly than Diclofenac (i.e. the binding affinity is ~ 40 to 90 fold higher) with a more favorable enthalpy change but a much lower binding stoichiometry (Table 1). Although the binding thermodynamics follow a similar overall trend, the large discrepancies in the reported literature values for the binding affinity, enthalpy and entropy changes for Naproxen binding to BSA might be related to the source of the protein and the drugs as well as the different experimental conditions employed in each study including buffer composition, ionic strength, and pH values. A recent analysis of several NSAID interactions with BSA and HSA using ITC and frontal analysis capillary electrophoresis (FA/CE) showed an effect of ionic strength and buffer on the thermodynamics of the interactions with up to 100-fold differences in the binding affinity in some cases [10]. Notably, under similar conditions [this work and ref. 10], the binding thermodynamics for the Naproxen-BSA interaction are in very good agreement (average values of $K \sim 2 \times 10^7 \text{ M}^{-1}$, $\Delta H \sim -58 \text{ kJ/mol}$, $\Delta S \sim -72 \text{ J/K.mol}$ at 25.00 °C, pH 7.40 and 50 mM ionic strength). Nonetheless, in all cases including the experimental conditions used in our study, Naproxen binding to BSA was much stronger than Diclofenac (in some cases the affinity constant was as much as 4 order of magnitude stronger) and showed a strongly enthalpically favored reaction that includes both electrostatic and hydrophobic contributions, as observed with Diclofenac (Table 1). Our data is consistent with earlier studies showing predominately electrostatic interactions between Naproxen and BSA as confirmed by the reduction of the binding affinity with increasing ionic strength and by competing and non-competing binding experiments in the presence of ionic and nonionic surfactants, respectively [12]. The higher enthalpic contributions observed with Naproxen binding to BSA suggest a more favorable number of hydrogen bonds and/or Van der Waals interactions compared to Diclofenac. The unfavorable entropy term ($T\Delta S$) suggests a more structured protein-drug complex (i.e. loss of conformational change upon complex formation and degrees of motional freedom, vibrational and/or rotational) as confirmed by the higher stability of the drug-protein complex determined by DSC (more below).

Figure 2: Calorimetric titrations of BSA and HSA with Diclofenac (A & C) and Naproxen (B & D), respectively. The insets are plots of the integrated heat versus the drug/protein molar ratios. Conditions: 30 μM protein solutions were titrated with 3 μL injections of 500 μM Diclofenac (A), 220 μM Naproxen (B), 900 μM Diclofenac (C), and 740 μM Naproxen (D). All solutions are in 50 mM phosphate, 50 mM NaCl, pH 7.40. The experimental pressure was 758 ± 6 mmHg.

T ($^{\circ}\text{C}$)	Binding Affinity K_a (m^{-1})	Enthalpy Change ΔH (kJ/mol)	Stoichiometry of Binding n	Dissociation Constant K_d (m)	Entropy Change ΔS (J/mol.K)	Gibbs Free Energy Change ΔG (kJ/mol)
BSA-Diclofenac						
20.00	$(2.49 \pm 1.48) \times 10^4$	-19.08 ± 3.48	1.88 ± 0.25	$(4.01 \pm 2.46) \times 10^{-5}$	18.87 ± 8.64	-24.63 ± 1.47
25.00	$(3.80 \pm 1.17) \times 10^4$	-31.95 ± 2.01	1.98 ± 0.11	$(2.62 \pm 0.82) \times 10^{-5}$	-19.64 ± 3.28	-26.13 ± 0.77
30.00	$(4.87 \pm 0.64) \times 10^4$	-39.28 ± 1.22	1.94 ± 0.05	$(2.05 \pm 0.27) \times 10^{-5}$	-42.67 ± 5.13	-27.19 ± 0.33
35.00	$(5.11 \pm 1.22) \times 10^4$	-45.01 ± 0.44	2.21 ± 0.33	$(1.95 \pm 0.47) \times 10^{-5}$	-55.34 ± 0.86	-27.77 ± 0.61
40.00	$(4.92 \pm 0.85) \times 10^4$	-52.51 ± 1.44	2.21 ± 0.32	$(2.02 \pm 0.35) \times 10^{-5}$	-77.40 ± 5.95	-28.13 ± 0.45
BSA-Naproxen						
20.00	$(2.00 \pm 0.89) \times 10^6$	-46.41 ± 1.72	0.69 ± 0.27	$(4.98 \pm 2.29) \times 10^{-7}$	-37.34 ± 9.75	-35.32 ± 1.10
25.00	$(1.72 \pm 0.79) \times 10^6$	-53.69 ± 7.38	0.811 ± 0.07	$(5.81 \pm 2.70) \times 10^{-7}$	-59.54 ± 6.53	-35.57 ± 1.15
30.00	$(2.02 \pm 0.36) \times 10^6$	-61.12 ± 3.27	0.67 ± 0.22	$(4.94 \pm 0.89) \times 10^{-7}$	-79.41 ± 3.11	-36.58 ± 0.45
35.00	$(1.78 \pm 0.99) \times 10^6$	-63.86 ± 2.33	0.74 ± 0.05	$(5.59 \pm 3.12) \times 10^{-7}$	-86.85 ± 2.62	-36.87 ± 1.43
40.00	$(1.64 \pm 0.72) \times 10^6$	-68.32 ± 1.22	0.82 ± 0.04	$(6.08 \pm 2.67) \times 10^{-7}$	-97.64 ± 1.47	-37.25 ± 1.14

Table 1: Best fit parameters for ITC measurements of Diclofenac and Naproxen binding to BSA. The experimental conditions are those of Figure 2 (i.e. 50 mM phosphate, 50 mM NaCl, pH 7.40). The Gibbs free energy of binding (ΔG) was calculated using the relationships $\Delta G = \Delta H - T\Delta S$. Standard errors (i.e. 0.68 confidence interval) from replicate determinations are indicated. The values of the binding affinity (K_a) in molarity are about 1.6 % lower than those expressed in molality at 20 °C and are essentially the same at 40 °C.

ITC of Diclofenac and Naproxen Binding to HSA

The bottom two panels of Figure 2 show the raw ITC data for Diclofenac and Naproxen titrations with HSA with the integrated heats for each injection shown in the insets following subtraction of control heats. The thermodynamic parameters are summarized in Table 2 and suggest a very different interaction compared to BSA. Binding of Diclofenac to HSA is both enthalpically and entropically favored suggesting that the interaction is due to hydrogen bonding reinforced by van der Waals hydrophobic effects (Table 2) whereas Naproxen binding to HSA showed three times higher binding stoichiometry compared to BSA (i.e. 3 Naproxen molecules bind per HSA compared to 1 Naproxen per BSA) with the binding interaction largely entropically favored (Table 2). The large and favorable changes in binding entropy observed with Naproxen binding to HSA are compensated by opposite changes in binding enthalpy resulting in an overall 10-fold lower binding affinity compared to BSA. In the case of HSA, the positive ΔS values can be attributed to the release of ordered water molecules from both the protein's binding site and the drug upon binding. Interestingly, no significant temperature effect was observed with either diclofenac or naproxen binding to HSA in the range of 20-40 °C. The average thermodynamic values for the binding of Diclofenac and Naproxen to HSA in that temperature range are as follows, $n = (2.13 \pm 0.13)$, $K = (4.13 \pm 2.03) \times 10^4 \text{ M}^{-1}$, $\Delta H = (-16.68 \pm 1.69) \text{ kJ/mol}$, $\Delta S = (32.85 \pm 6.17) \text{ J/K.mol}$ for Diclofenac and $n = (3.05 \pm 0.21)$, $K = (1.24 \pm 0.19) \times 10^5 \text{ M}^{-1}$, $\Delta H = (-12.89 \pm 1.65) \text{ kJ/mol}$, $\Delta S = (54.96 \pm 5.16) \text{ J/K.mol}$ for Naproxen, respectively (Table 2).

T (°C)	Binding Affinity K_a (M ⁻¹)	Enthalpy Change ΔH (kJ/mol)	Stoichiometry of Binding n	Dissociation Constant K_d (M)	Entropy Change ΔS (J/mol.K)	Gibbs Free Energy Change ΔG (kJ/mol)
HSA-Diclofenac						
20.00	$(2.31 \pm 1.17) \times 10^4$	-15.18 ± 1.77	2.13 ± 0.32	$(4.34 \pm 2.27) \times 10^{-5}$	31.62 ± 7.00	-24.45 ± 1.25
25.00	$(3.18 \pm 1.42) \times 10^4$	-18.57 ± 2.03	2.07 ± 0.09	$(3.14 \pm 1.42) \times 10^{-5}$	23.87 ± 5.91	-25.68 ± 1.89
30.00	$(7.29 \pm 4.36) \times 10^4$	-18.35 ± 4.63	1.97 ± 0.49	$(1.36 \pm 0.83) \times 10^{-5}$	34.01 ± 6.99	-28.66 ± 1.86
35.00	$(5.05 \pm 0.64) \times 10^4$	-15.07 ± 0.54	2.33 ± 0.46	$(1.97 \pm 0.25) \times 10^{-5}$	41.12 ± 2.79	-27.74 ± 0.32
40.00	$(2.92 \pm 0.32) \times 10^4$	-16.23 ± 1.45	2.15 ± 0.21	$(3.42 \pm 0.37) \times 10^{-5}$	33.66 ± 3.71	-26.77 ± 0.29
HSA-Naproxen						
20.00	$(1.16 \pm 0.85) \times 10^5$	-10.60 ± 2.19	2.82 ± 0.25	$(8.63 \pm 6.54) \times 10^{-6}$	61.13 ± 6.82	-28.38 ± 1.82
25.00	$(1.52 \pm 0.43) \times 10^5$	-14.67 ± 1.20	3.17 ± 0.16	$(6.58 \pm 1.88) \times 10^{-6}$	50.16 ± 6.37	-29.56 ± 1.25
30.00	$(1.04 \pm 0.29) \times 10^5$	-14.30 ± 0.66	3.11 ± 0.04	$(9.58 \pm 2.68) \times 10^{-6}$	49.05 ± 3.63	-29.11 ± 0.71
35.00	$(1.15 \pm 0.41) \times 10^5$	-12.25 ± 1.93	2.86 ± 0.09	$(8.66 \pm 3.10) \times 10^{-6}$	57.54 ± 3.23	-29.85 ± 0.91
40.00	$(1.36 \pm 0.26) \times 10^5$	-12.64 ± 0.95	3.31 ± 0.38	$(7.34 \pm 1.41) \times 10^{-6}$	56.91 ± 6.17	-30.77 ± 0.49

Table 2: Best fit parameters for ITC measurements of Diclofenac and Naproxen binding to HSA. The experimental conditions are those of Figure 2 (i.e. 50 mM phosphate, 50 mM NaCl, pH 7.40). The Gibbs free energy of binding (ΔG) was calculated using the relationships $\Delta G = \Delta H - T\Delta S$. Standard errors (i.e. 0.68 confidence interval) from replicate determinations are indicated. The values of the binding affinity (K_a) in molarity are about 1.6 % lower than those expressed in molality at 20 °C and are essentially the same at 40 °C.

Numerous biophysical techniques and indirect methods have yielded inconsistent results for the affinity and the number of Diclofenac and Naproxen binding sites on serum albumins with some reports showing one set of strong binding sites while others reporting two sets of sites with high and low binding affinities [10, 11 and refs therein]. To our knowledge, no direct measurement is reported in the literature of Diclofenac interaction with HSA and only one other direct study (beside this work) has examined Naproxen binding to HSA using ITC [10]. In that latter study, the authors reported the presence of two consecutive binding events of Naproxen with HSA with high ($K = 9 \times 10^5 \text{ M}^{-1}$) and low ($K \sim 7 \times 10^4 \text{ M}^{-1}$) affinity constants and binding stoichiometries of 1 and 2.5, respectively. Notably, using FA/CE, the authors found in the same study that the interaction of Naproxen with HSA exhibited one weak class of binding sites ($n = 3.5$) with a binding affinity that is similar to that determined by ITC (i.e. $K = 7.40 \times 10^4 \text{ M}^{-1}$); however the CE data is too scattered to be reliable. Our Naproxen-HSA ITC data is in agreement with a

recent study using fluorescence and phosphorescence spectroscopy in the steady-state and time-resolved mode [32], showing one class of strong binding sites. No significant differences in the binding constants of two Naproxen enantiomers (S)-NPX and (R)-NPX interaction with HSA (i.e. $K \sim 3 \times 10^5 \text{ M}^{-1}$ for both enantiomers) were observed. Binding constants of the same order of magnitude have also been reported in earlier studies [33, 34]. As to Diclofenac interaction with HSA, it is almost impossible to directly compare thermodynamic values given the inconsistency in the literature data and the techniques used, with some studies reporting single binding sites with K values in the range of 5×10^3 to $5 \times 10^5 \text{ M}^{-1}$, while others showing two binding sites, with high and low binding affinities ranging between 3.0×10^3 and $3 \times 10^6 \text{ M}^{-1}$ [11 and references therein]. Collectively, what is clear is that the second class of non-specific and weak binding sites appears to be dependent on the sensitivity of the technique used as well as the experimental conditions under which the experiments were performed.

Despite the high similarity between human and bovine serum albumins, the two proteins display different binding properties as reported by numerous binding studies and X-ray crystallographic data. While Sudlow sites I and II located in subdomains IIA and IIIA are the most common and major binding sites for many drugs, including Naproxen and Diclofenac, other potential drug binding sites that have been proposed or identified via crystal structures are located in subdomains IB, IIIA and IIIB. Interestingly, no binding sites in subdomains IA and IIB have been observed. Based on available spectroscopy and crystallographic data, we can identify (or predict) that Naproxen binding to HSA occurs at subdomains IIA, IIIA and IB (i.e. 3 binding sites or 3 Naproxen molecules per HSA), that Diclofenac binding to HSA takes place at subdomains IB and IIA (i.e. 2 binding sites or 2 Diclofenac molecules per HSA), that Naproxen binding to BSA is likely to be in subdomain IIIA (i.e. < 1 binding site or ~ 0.75 Naproxen molecule per BSA), and finally that Diclofenac binding to BSA occurs at subdomains IIIA, IIA or possibly IIIB (i.e. 2 binding sites or 2 Diclofenac molecules per BSA) [27, 35-37].

Amongst all the interactions studied here, Naproxen binding to BSA exhibited a binding stoichiometry close to unity with a strong binding affinity and a high binding enthalpy of reaction (Table 1). In support of this finding, a recent X-ray study [35] showed stabilization of a Naproxen-BSA complex by a combination of hydrophobic and hydrogen bonding contacts between the naphthalene ring of the drug and the leucines of subdomain IIIA, the carboxyl group

of the drug and residues Tyr410, Lys413, Arg409, and Ser488 located at the entrance of subdomain IIIA, respectively. The thermodynamic parameters for the other drug-protein interactions (Diclofenac-HSA, Diclofenac-BSA, and Naproxen-HSA) are average values from drugs binding to multiple sites on the protein and are weaker/smaller than those of Naproxen binding to BSA. Nonetheless, these interactions still consisted of both hydrophobic contacts (between the hydrophobic groups of the drug molecules and the different hydrophobic pockets of the subdomains) and hydrogen bondings (between the carboxylate groups of the drugs and several surrounding residues) [27, 35-37].

Heat Capacity Change and Enthalpy–Entropy Compensation

In ligand binding, the term “entropy-enthalpy compensation” usually means that a change in ligands results in a change in enthalpy of binding which could be partially or fully offset by a change in the entropy of binding such as $\Delta\Delta H \approx T\Delta\Delta S$ and the net change in binding affinity $\Delta\Delta G \approx 0$. Evidence of compensation is often presented in literature in the form of a graph in which $T\Delta S$ is plotted against ΔH and fit with a linear regression often with a slope near unity. The changes of our thermodynamic parameters as a function of temperature suggest an enthalpy–entropy compensation phenomenon as indicated by the linear relationship of a plot of $T\Delta S$ vs. ΔH using the equation $T\Delta S = \alpha\Delta H + \beta$ where the slope α and the Y-intercept β are equal to 0.87 and 21.8 for Diclofenac binding to BSA, and 0.901 and 30.7 for Naproxen binding to BSA (Fig. 3A). To corroborate that this enthalpy–entropy compensation effect is due to weak non-covalent interactions as previously reported [38, 39], a plot of ΔG vs ΔH using the relationship $\Delta G = (1-\alpha)\Delta H - \beta$ yielded very similar α and β values of 0.899 and 23.1 for Diclofenac-BSA interaction and 0.907 and 30.8 for Naproxen-BSA interaction, respectively (Fig. 3B). In all cases, the slope is close to unity (i.e. α values of 0.901 and 0.899) indicating almost complete enthalpy–entropy compensation; note that the free energy of binding remained almost unchanged despite large observed changes in ΔH and ΔS . The decrease in the entropy of binding with an increase in temperature (i.e. larger negative values of ΔS as T increases, Table 1) is also consistent with a more rigid drug-protein complex as a result of binding.

Figure 3: Plots of enthalpy-entropy compensation, (A) $T\Delta S$ versus ΔH and (B) ΔG versus ΔH for the binding of Diclofenac and Naproxen to BSA at pH 7.40 and 25 °C. (C) Plot of the enthalpy change against temperature for the determination of the heat capacity change (ΔC_p). The conditions are those of Figure 2 and the data is that of Tables 1 and 2.

The heat capacity change (ΔC_p) is determined from the temperature dependence of the binding enthalpy using the relationship $\Delta C_p = \delta(\Delta H) / \delta T$ and can provide insights into structure-energetics relationships pertaining to solvent reorganization (i.e. hydration-dehydration events) accompanying drug-protein interactions. Figure 3C illustrates the temperature dependence of ΔH associated with the binding of Diclofenac and Naproxen to BSA. In either case, excellent linear fits with correlation coefficients R of 0.990 for Naproxen and 0.996 for Diclofenac were obtained with ΔC_p values (slope of the lines, $\Delta\Delta H / \Delta T$) of -1.03 kJ/K.mol for Naproxen and -1.41 kJ/K.mol for Diclofenac (Fig. 3C). These ΔC_p values are similar to those found with other drugs binding to serum albumins, drug-DNA and protein-ligand interactions, and are generally attributed to burial of hydrophobic groups, the formation of a more compact drug-protein complex, and loss of water molecules due to binding [12, 38, 40, 41].

Fluorescence Spectroscopy

Many drugs (including NSAIDs) have been shown to bind to serum albumins and quench the fluorescence of tryptophan residues via a static quenching mechanism [32, 28, 42-45] whereby non-fluorescent ground-state fluorophore-quencher complexes form. Dynamic quenching (also referred to as collisional quenching) is another type of molecular quenching whereby a quencher collides with a fluorophore in an excited state followed by non-radiative transitions to the ground state. Dynamic and static quenching differ in their dependence on temperature and viscosity; higher temperatures will result in faster diffusion rates and hence larger number of collisional

quenching (i.e. larger bimolecular quenching constants at higher temperature), whereas increasing temperatures will typically reduce the stability of the fluorophore-quencher complexes and result in a lower static quenching constant [46]. The binding of Diclofenac to BSA and HSA was monitored by following the intrinsic fluorescence of the proteins at 346 nm using an exciting wavelength of 280 nm. Naproxen binding to either BSA or HSA could not be followed by fluorescence spectroscopy due to Naproxen's own fluorescence emission spectrum at 346 nm which overlaps with that of the protein. Nonetheless, earlier fluorescence quenching measurements showed that Naproxen binding to BSA caused minimal change in the emission spectra of the protein with an initial fluorescence decrease at low drug concentration (due to binding) followed by an increase in the fluorescence intensity at high drug concentration that is likely due to the fluorescence emission of Naproxen itself [12].

Figure 4 shows significant quenching of the fluorescence emission spectra at 25 °C of BSA and HSA with increasing drug concentrations, suggesting binding and substantial alteration in the local environment of the tryptophan moieties in or around the proteins' binding sites (i.e. Trp-134 and Trp-212 in BSA and Trp-214 in HSA).

Figure 4: Change in the fluorescence emission spectra of HSA (A) and BSA (B) in the presence of various concentrations of Diclofenac at 25 °C in 50 mM phosphate, 50 mM NaCl, pH 7.40. The spectra were collected by injecting 3 μ l of 625 μ M Diclofenac solution into 10 μ M protein solution. The excitation wavelength was set at 280 nm and the excitation/emission slits were both at 5 nm. The insets show the percent quenching as a function of drug concentration.

To clarify the type of fluorescence quenching mechanism induced by human and bovine serum albumins, the fluorescence data was analyzed according to the Stern–Volmer equation and is plotted in Fig. 5:

$$\frac{I_0}{I} = 1 + k_q \tau_0 [D] = 1 + K_{sv} [D]$$

where I_0 and I are the intensities of the fluorescence before and after addition of Diclofenac, k_q the bimolecular quenching rate constant, τ_0 the average lifetime of the fluorophore in the absence of the quencher (~ 5 ns), K_{sv} the Stern–Volmer bimolecular quenching constant, and $[D]$ the concentration of the drug (46). We note that this equation predicts a linear relation between I_0/I and $[D]$ with an intercept of 1 and does not necessarily discriminate between dynamic or static quenching. Thus, one cannot determine whether quenching is static or dynamic only on the basis of Stern–Volmer linearity, however, if such plots are obtained as a function of temperature (Fig. 5), a determination from the change in slope might be possible. If the slope increases with increasing temperature, one would conclude that the quenching is dynamic, but if the slope decreases with increasing temperature then the quenching is said to be static.

Figure 5: Stern–Volmer plots of I_0/I vs. Diclofenac concentrations for HSA and BSA at different temperatures. The conditions are the same as those in Figure 4.

The parameters of the Stern–Volmer plots for HSA and BSA quenching by Diclofenac at different temperatures are summarized in Table 3. Because K_{sv} decreased with increasing temperature and k_q is greater than the maximum scatter collision quenching constant of various quenchers with biopolymers ($k_q \sim 2.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), the quenching is therefore not initiated by dynamic collision but rather by static collision through the formation of ground-state protein-drug complex (Table 3).

T (K)	Stern–Volmer Bimolecular Quenching Constant $K_{sv} \times 10^{-5} \text{ (m}^{-1}\text{)}$	R^2	Bimolecular Quenching Rate Constant $kq \times 10^{-13} \text{ (m}^{-1}\text{s}^{-1}\text{)}$
BSA-Diclofenac			
293.15	1.12 ± 0.19	0.99558	2.23
298.15	1.09 ± 0.17	0.99399	2.17
303.15	1.06 ± 0.08	0.99532	2.13
308.15	0.97 ± 0.07	0.99603	1.94
313.15	0.96 ± 0.02	0.99511	1.92
HSA-Diclofenac			
293.15	2.19 ± 0.12	0.99357	4.39
298.15	1.97 ± 0.23	0.99245	3.94
303.15	1.65 ± 0.07	0.99337	3.29
308.15	1.62 ± 0.01	0.99505	3.25
313.15	1.41 ± 0.09	0.99559	2.82

Table 3: Stern-Volmer quenching constants for the binding of Diclofenac to BSA and HSA in the temperature range of 293.15 to 313.15 K, in 50 mM phosphate buffer, 50 mM NaCl, pH 7.40. The experimental pressure was 758 ± 6 mmHg. The values of K_{sv} and kq in molarity are about 1.6 % lower than those expressed in molality at 20 °C and are essentially the same at 40 °C.

Differential Scanning Calorimetry (DSC)

DSC experiments were performed to examine the effect of drug binding on the melting temperature and thermal stability of serum albumins. Figure 6 illustrates the DSC profiles for BSA and HSA in the absence and presence of Diclofenac and Naproxen, and Table 4 summarizes the denaturation parameters of these interactions in 50 mM phosphate buffer, 50 mM NaCl, pH 7.40. The DSC data depict a strong stabilization effect upon drug binding with a significant increase in the melting temperatures of the protein-drug complexes (Table 4). Several unfolding events for BSA and HSA in the absence of drugs are observed in the range of 60 – 80 °C with transition enthalpies ranging between 334 – 707 kJ/mol (Table 4). Binding of various molar ratios of Diclofenac and Naproxen to all samples caused a significant shift of the melting temperature T_m to higher temperatures and is attributed to the unfolding of the drug-protein complexes (Figure 6). In the case of BSA, four distinct transition peaks were observed in the absence of drugs at about 60, 65, 75 and 81 °C which can be attributed to the sequential unfolding of the different domains found in serum albumins. At 2:1 molar ratios of Diclofenac and Naproxen to BSA, an average increase of 7 °C, 5.5 °C and 2 °C in the melting temperature T_m is observed, respectively, with the shoulder at 81 °C unaffected by the presence of such low drug to protein molar ratios. However, at higher drug to protein molar ratios of 10:1, only two transitions are shown with a significant increase in the melting points of 18 and 15 °C,

respectively. The transition temperatures for Diclofenac and Naproxen bound to HSA showed a more modest increase of 0.5 °C to 4°C for the first three transitions at low drug to protein molar ratios of 1:1 and 1:2 (Table 4). At higher protein to drug molar ratios of 1:10, only two transitions were observed with an average increase of 14°C compared to drug-free protein (Table 4). The DSC thermograms of all samples examined here exhibit multimodal behavior that can be attributed to the sequential thermal unfolding of the different domains present or large structural modifications of protein's secondary and tertiary structures. The DSC data for the unfolding of BSA and BSA-drug complexes have some elements that are in good agreement with data published with other NSAIDs [28, 47]. The discrepancies in some of the observed values (Table 5) might be due to different parameters including source, type (fatty acid bound- or fatty acid-free protein), purity of the protein and drug, as well as different experimental conditions employed in each study such as buffer composition, ionic strength, and pH. Overall, the DSC results provided a quantitative measure of the thermal stability of the protein in the presence of Diclofenac and Naproxen and showed that drugs induced stabilization effects are significantly larger in the case of BSA consistent with a tighter binding as observed by ITC (Tables 1, 2 and 4).

Figure 6: DSC curves of BSA and HSA in the absence or in the presence of various molar ratios of Diclofenac or Naproxen to protein (solid black lines) as indicated on each panel. The dotted lines represent fit to the experimental solid curves (i.e. model sum of the green, blue, magenta and brown lines). Conditions: 10 μ M protein solutions, 50 mM phosphate, 50 mM NaCl, pH 7.40. The experimental pressure was 3.00 ± 0.03 atm.

Protein-Drug (ratio)	<i>Enthalpy Change of Unfolding</i> ΔH_1(kJ/mol)	<i>Melting Temp</i> T_{m1}(°C)	<i>Enthalpy Change of Unfolding</i> ΔH_2(kJ/mol)	<i>Melting Temp</i> T_{m2}(°C)	<i>Enthalpy Change of Unfolding</i> ΔH_3(kJ/mol)	<i>Melting Temp</i> T_{m3}(°C)	<i>Enthalpy Change of Unfolding</i> ΔH_4(kJ/mol)	<i>Melting Temp</i> T_{m4}(°C)
HSA (no drug)	634.9	62.0	450.9	65.5	413.2	71.7	632.6	76.7
HSA-Naproxen (1:1)	598.3	65.0	444.4	69.3	483	75.4	---	---
HSA-Naproxen (1:10)	882.1	76.4	650.5	79.0	---	---	---	---
HSA-Diclofenac (1:2)	632.1	64.2	544.2	67.6	411.1	72.3	---	---
HSA-Diclofenac (1:10)	669.8	76.8	1049	79.1	---	---	---	---
BSA (no drug)	376.2	59.7	334.1	65.4	408.8	74.9	707	80.9
BSA-Naproxen (1:2)	447.3	66.6	467.2	70.9	651.3	76.7	802.8	80.9
BSA-Naproxen (1:10)	852.2	77.8	1423	80.9	---	---	---	---
BSA-Diclofenac (1:2)	480.4	66.7	564.2	71.4	603.1	76.7	827	81.0
BSA-Diclofenac (1:10)	988.7	77.6	1059	80.4	---	---	---	---

Table 4: DSC results for the thermal unfolding of 1 mg/ml BSA and HSA in the absence or in the presence of various molar ratios of Diclofenac or Naproxen to protein, as indicated. Subscripts 1, 2, 3 and 4 for the melting temperatures (T_m) and the enthalpy change of unfolding (ΔH) represent the different components of the deconvoluted DSC endotherms. The experimental conditions are those of Figure 6 and the reported values of T_m and ΔH are average values from multiple runs that typically differ by 10% or less from run to run.

<i>Protein-Drug (ratio)</i>	<i>Enthalpy Change of Unfolding ΔH_1(kJ/mol)</i>	<i>Melting Temp T_{m1}(°C)</i>	<i>Enthalpy Change of Unfolding ΔH_2(kJ/mol)</i>	<i>Melting Temp T_{m2}(°C)</i>	<i>Enthalpy Change of Unfolding ΔH_3(kJ/mol)</i>	<i>Melting Temp T_{m3}(°C)</i>	<i>Enthalpy Change of Unfolding ΔH_4(kJ/mol)</i>	<i>Melting Temp T_{m4}(°C)</i>	<i>Ref</i>
<i>Fatty Acid Bound BSA (Drug free)</i>	376.2	59.7	334.1	65.4	408.8	74.9	707	80.9	<i>This work</i>
<i>Fatty Acid Bound BSA-Diclofenac (1:2)</i>	480.4	66.7	564.2	71.4	603.1	76.73	827	81.0	<i>This work</i>
<i>Fatty Acid Bound BSA-Diclofenac (1:10)</i>	988.7	77.6	1059	80.4	---	---	---	---	<i>This work</i>
<i>Fatty Acid Bound BSA (Drug Free)</i>	379	62.8	---	---	---	---	825	80.9	[47]
<i>Fatty Acid Bound BSA-Diclofenac (1:22)</i>	---	---	---	---	623	75.7	1672	83.7	[47]
<i>Fatty Acid Free BSA (Drug Free)</i>	452	61.8	---	---	---	---	---	---	[47]
<i>Fatty Acid Free BSA-Diclofenac (1:22)</i>	---	---	---	---	701	76.1	---	---	[47]
<i>Fatty Acid Free BSA-Diclofenac (1:2)</i>	299.7	61.5	364.5	67.9	---	---	---	---	[28]
<i>Fatty Acid Free BSA-Diclofenac (1:7)</i>	---	---	---	---	480.7	72.3	---	---	[28]

Table 5: Comparison of DSC results (T_m , melting temperatures and ΔH , enthalpy change of unfolding) for the thermal unfolding of BSA in the absence and presence of various molar ratios of Diclofenac to protein. For detailed experimental conditions, the reader is encouraged to check the appropriate references.

Conclusion

Human serum albumin-drug complexes are considered dynamic systems from which drugs are released and metabolized. Binding of drugs occurs at specific sites on the protein and is a major determinant in drug absorption, distribution, bioavailability, metabolism and toxicity. Notably, HSA can also be used as a biomarker of many diseases, including cancer and diabetes [48].

The quantitative analysis presented here provides a deeper understanding of the driving forces, structural and dynamic changes that occur between serum albumins and two NSAID molecules, Diclofenac and Naproxen. Knowledge of the nature and magnitude of these interactions is critical in evaluating the pharmacokinetic and pharmacodynamic properties of drugs during drug design and development. Our data showed that the binding of Diclofenac and Naproxen to BSA is enthalpically favored and is mostly electrostatic in nature with some hydrophobic effects. The unfavorable entropy term suggests a more structured protein-drug complex as confirmed by DSC. The Diclofenac-BSA fluorescence quenching data suggest static collisions and the formation of a ground-state protein-drug complex. With HSA, the binding of the two drugs is both enthalpically and entropically favored suggesting hydrogen bonds formation reinforced by considerable van der Waals hydrophobic effects. A significant enthalpy–entropy compensation phenomenon was observed with Diclofenac and Naproxen binding to BSA but not to HSA where no significant temperature effect was reported in the temperature range of 20-40 °C employed in this study. Interestingly, it has been proposed that entropy-enthalpy compensation should be a major concern during lead optimization in drug discovery in light of drug resistance mutations that could introduce compensatory changes in the enthalpies and entropies of inhibitor binding leading to significant changes in ligand recognition mechanisms [49]. Given that binding of Diclofenac and Naproxen to BSA is strongly enthalpically favored with a strong unfavorable entropy change, the almost perfect enthalpy-entropy compensation observed with BSA could be attributed to the reorganization of hydrogen bonds from lost water molecules upon binding resulting in little change in net free energy (Table 1). Finally, the multimodal DSC thermograms reflect the sequential unfolding of different protein domains which are shown to exhibit strong stabilization effects by high protein drug molar ratios.

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