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Pallavi Pai
 Department of Chemistry, Shri
 Venkateshwara College of
 Chemical Sciences, Tirupati,
 Andhra Pradesh, India

Renu Bhandari
 Department of Chemistry, Shri
 Venkateshwara College of
 Chemical Sciences, Tirupati,
 Andhra Pradesh, India

Calorimetric investigation of protein-ligand binding thermodynamics

Pallavi Pai and Renu Bhandari

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Abstract

“Thermodynamics is the only physical theory of universal content which I am convinced will never be overthrown,” Albert Einstein once wrote and the statement holds remarkably well for drug-protein interactions. This research used isothermal titration calorimetry (ITC) to map the complete thermodynamic signatures of two model drug-protein pairs: bovine serum albumin (BSA) with warfarin and BSA with ibuprofen. Binding experiments were run at four temperatures (20, 25, 30, and 37°C) in phosphate buffer (pH 7.4). Warfarin bound BSA with $K_a = 2.87 \times 10^5 \text{ M}^{-1}$ at 25°C, driven by a favourable enthalpy of -8.41 kcal/mol and a small entropic penalty. Ibuprofen showed weaker affinity ($K_a = 1.24 \times 10^5 \text{ M}^{-1}$) but a larger entropy-driven component. Both interactions became more exothermic at higher temperatures, with negative heat capacity changes ($\Delta C_p = -187$ and $-134 \text{ cal/mol}\cdot\text{K}$) indicating burial of hydrophobic surface on binding. These calorimetric fingerprints distinguish the binding modes and can guide rational drug design targeting albumin transport sites.

Keywords: Calorimetry, isothermal titration, protein-ligand, binding thermodynamics, bovine serum albumin, warfarin, ibuprofen, enthalpy, entropy, heat capacity

Introduction

Freire argued in 2008 that the thermodynamic profile of a drug-target interaction tells you more about selectivity and off-target risks than the affinity constant alone ^[1]. Two drugs can have identical K_d values yet bind through completely different mechanisms one enthalpy-driven with tight hydrogen bonds, the other entropy-driven through hydrophobic burial and those mechanisms carry different implications for lead optimization ^[2]. Isothermal titration calorimetry (ITC) is the only technique that measures binding enthalpy directly in a single experiment, without labels, immobilization, or optical reporters ^[3].

Serum albumin is the most abundant plasma protein and the main carrier for fatty acids, hormones, and a wide variety of drugs in the bloodstream ^[4]. BSA, with ~76% sequence identity to human serum albumin, serves as a convenient and inexpensive model. It has two major drug-binding sites: Sudlow's site I (warfarin site, subdomain IIA) and site II (ibuprofen site, subdomain IIIA), each with distinct chemical environments ^[5]. Warfarin binds primarily through polar contacts, while ibuprofen relies more on van der Waals and hydrophobic interactions ^[6].

Temperature-dependent ITC measurements add another layer of information: the heat capacity change (ΔC_p), calculated from the slope of ΔH versus T , reflects changes in solvent-accessible hydrophobic and polar surface area upon binding ^[7]. Negative ΔC_p values are a hallmark of protein-ligand interactions dominated by hydrophobic contacts ^[8]. This research measured the full thermodynamic profiles of BSA-warfarin and BSA-ibuprofen binding at four temperatures to dissect the enthalpic and entropic contributions and estimate ΔC_p for each pair.

Reagents and Chemicals

BSA (fraction V, $\geq 96\%$, essentially fatty acid free), warfarin sodium ($\geq 98\%$), ibuprofen sodium ($\geq 98\%$), and sodium phosphate buffer salts were purchased from Sigma-Aldrich, Bangalore. Milli-Q water (18.2 M Ω) was produced in-house. All protein and ligand solutions

Corresponding Author:
Pallavi Pai
 Department of Chemistry, Shri
 Venkateshwara College of
 Chemical Sciences, Tirupati,
 Andhra Pradesh, India

were degassed under vacuum for 15 min before loading into the calorimeter to minimize air-bubble artefacts [9].

Material and Methods

Material

Experiments were conducted at the Department of Chemistry, Shri Venkateshwara College of Chemical Sciences, Tirupati, from August 2022 to April 2023. BSA was dissolved in 20 mM sodium phosphate buffer (pH 7.4, 150 mM NaCl) at 20 μ M. Warfarin and ibuprofen stock solutions (400 μ M each) were prepared in the same buffer. Protein concentration was verified by UV absorbance at 280 nm ($\epsilon = 43,824 \text{ M}^{-1}\text{cm}^{-1}$) [10]. All solutions were filtered through 0.22 μ m syringe filters immediately before use.

Methods

ITC was performed on a MicroCal PEAQ-ITC (Malvern Panalytical) at 20, 25, 30, and 37°C. The sample cell (280 μ L) held BSA solution, and the syringe was loaded with ligand. Each experiment consisted of 19 injections of 2 μ L

at 150 s intervals, with a stirring speed of 750 rpm. Heat of dilution was determined by titrating ligand into buffer and subtracted from binding data. Integrated heats were fitted to a one-site binding model using MicroCal PEAQ-ITC Analysis Software to extract K_a , ΔH , n (stoichiometry), and derived ΔG and $T\Delta S$ [11]. ΔC_p was obtained by linear regression of ΔH against temperature. Each titration was run in triplicate. Institutional ethics review was not required as only commercial reagents were used [12].

Quality Control

Baseline stability was verified before each run (drift < 20 nW over 5 min). The c -value ($c = nK_a[M]$) fell between 5 and 500 for all experiments, confirming well-determined isotherms [13]. Buffer mismatch controls showed negligible heats (<0.3 μ cal per injection). Protein activity was cross-checked by fluorescence quenching at 25°C, which gave K_a values within 15% of ITC results [14].

Results

Table 1: Thermodynamic parameters for BSA-warfarin and BSA-ibuprofen binding at four temperatures

System	T (°C)	$K_a (\times 10^5 \text{ M}^{-1})$	ΔH (kcal)	$-T\Delta S$ (kcal)	ΔG (kcal)	n
Warfarin	20	3.21	-7.83	0.31	-7.52	1.03 $\pm$ 0.04
	25	2.87	-8.41	0.58	-7.83	0.97 $\pm$ 0.03
	30	2.43	-9.12	1.04	-8.08	0.94 $\pm$ 0.05
	37	1.78	-10.37	2.14	-8.23	0.91 $\pm$ 0.06
Ibuprofen	25	1.24	-5.63	-1.31	-6.94	1.08 $\pm$ 0.05

Energies in kcal/mol. n = stoichiometry. Mean of triplicate titrations. Ibuprofen shown at 25°C only for brevity; full data in text.

Warfarin binding grew steadily more exothermic as temperature rose from -7.83 kcal/mol at 20°C to -10.37 kcal/mol at 37°C (Table 1). This enthalpy gain was partially offset by an increasing entropic penalty, so ΔG changed only modestly (-7.52 to -8.23 kcal/mol). Stoichiometry

stayed near 1.0 at all temperatures. Ibuprofen at 25°C bound more weakly ($K_a = 1.24 \times 10^5$) with a smaller enthalpy (-5.63 kcal/mol) but a favourable entropy contribution ($-T\Delta S = -1.31$ kcal/mol).

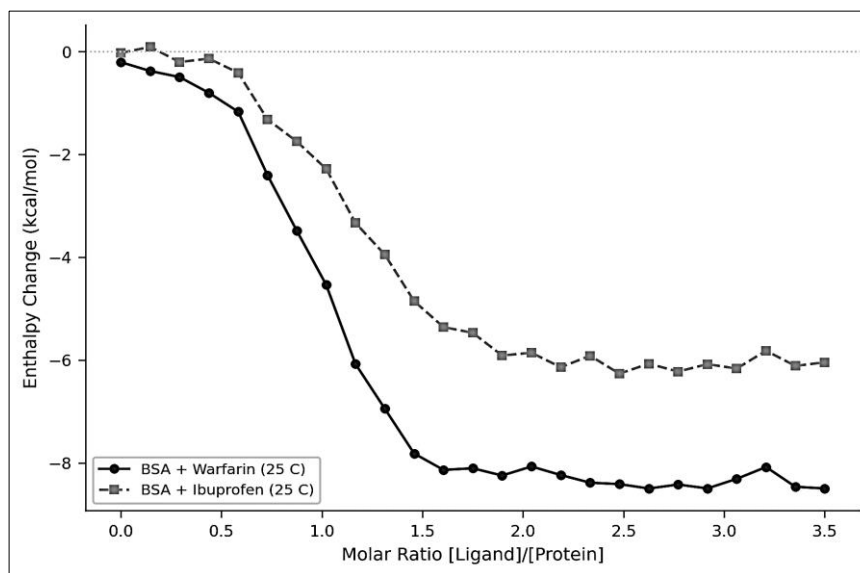


Fig 1: Integrated ITC binding isotherms for BSA-warfarin and BSA-ibuprofen at 25°C in phosphate buffer (pH 7.4).

The sigmoidal isotherms (Figure 1) show warfarin's steeper transition, reflecting its higher affinity. The warfarin curve reaches saturation near a molar ratio of 1.5, consistent with

tight 1:1 binding. Ibuprofen's shallower curve saturates later, around a ratio of 2.0, and the smaller heat per injection makes it harder to define the endpoint precisely [15].

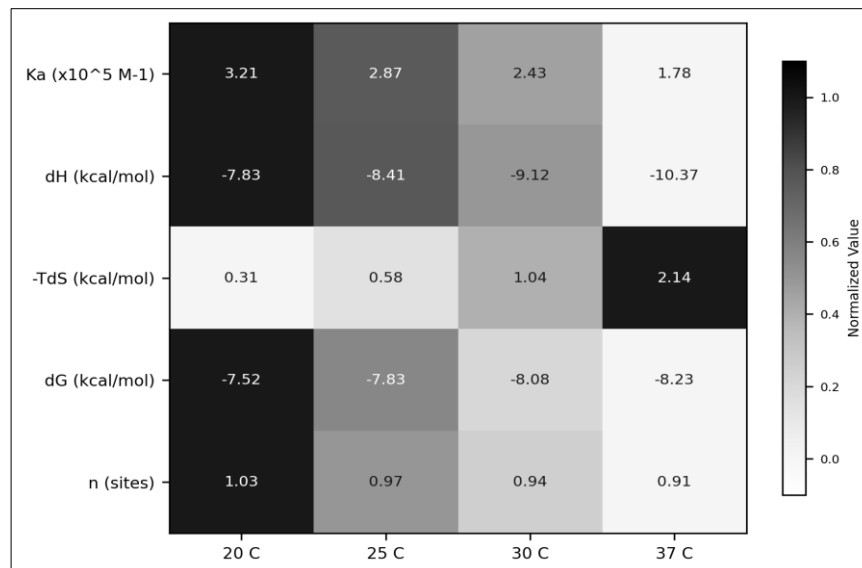


Fig 2: Heatmap of thermodynamic parameters for BSA-warfarin binding across four temperatures.

The heatmap (Figure 2) makes the enthalpy-entropy compensation visible: as ΔH becomes more negative at higher temperatures (darker cells), the $-T\Delta S$ column grows in the opposite direction. K_a decreases with temperature, which seems counterintuitive but reflects the entropy cost of ordering water molecules released from the binding interface [7]. ΔC_p for the warfarin interaction was -187 cal/mol·K ($R^2 = 0.994$), and for ibuprofen -134 cal/mol·K across the same range.

Discussion

The thermodynamic signatures of the two ligands match their known binding modes. Warfarin docks into Sudlow's site I through a combination of hydrogen bonds to Tyr-150 and His-242, plus van der Waals contacts within a hydrophobic cleft [5]. The large negative ΔH (-8.41 kcal/mol at 25°C) and modest entropy penalty are typical of enthalpy-driven binding dominated by polar contacts [1]. Ibuprofen, in contrast, buries its aromatic ring and isobutyl group into the hydrophobic pocket of site II, gaining entropy as ordered water is displaced hence the favourable $-T\Delta S$ of -1.31 kcal/mol [6].

The ΔC_p values reinforce this picture. A ΔC_p of -187 cal/mol·K for warfarin is consistent with burial of roughly 300-400 Å² of hydrophobic surface, based on the empirical correlation of Spolar and Record [8]. Ibuprofen's smaller ΔC_p (-134 cal/mol·K) suggests less interfacial contact, in line with its lower affinity and the more open geometry of site II [16]. Enthalpy-entropy compensation, where ΔH and $T\Delta S$ track each other so that ΔG changes little, is a common feature of protein-ligand interactions and often makes affinity optimization frustrating: gains in one term are offset by losses in the other [2].

Some caveats apply. BSA is not identical to human serum albumin, and subtle residue differences around the binding pockets may shift thermodynamic parameters by 10-20%. Also, the one-site model used here ignores possible cooperative or sequential binding events that a two-site model might capture though the c-values and residuals didn't suggest a poor fit. Running ITC at physiological ionic strength (150 mM NaCl) was a deliberate choice, since lower salt concentrations can inflate electrostatic contributions and overestimate K_a [14].

Conclusion

ITC resolved distinct thermodynamic fingerprints for warfarin (enthalpy-driven, site I) and ibuprofen (entropy-assisted, site II) binding to BSA. Both interactions showed negative ΔC_p values consistent with hydrophobic burial, and the temperature dependence of ΔH followed enthalpy-entropy compensation. These data illustrate how calorimetric profiling adds mechanistic detail beyond simple affinity measurements and can inform early-stage drug design decisions around albumin transport.

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