ON THE INTERACTIONS BETWEEN WARFARIN AND HUMAN (HSA) OR BOVINE (BSA) SERUM ALBUMIN EVALUATED BY DIFFERENT ANALYTICAL TECHNIQUES

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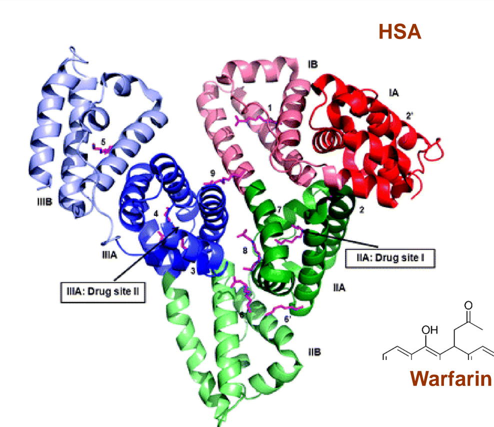
INTRODUCTION

Albumin, the most abundant protein in plasma and serum, is a water-soluble macromolecule with high biological significance. It is able to maintain the plasma oncotic pressure and modulate the fluid distribution among body compartments, showing also considerable buffering, antioxidant and pseudo-enzymatic abilities. Native albumin, is built up from three homologous domains (I, II and III), showing each one two distinct subdomains, named A and B. There are numerous binding sites on albumin, but drugs and other exogenous compounds bind, mainly, two of them: Sudlow I or acidic drug binding site, placed on subdomain IIA, and Sudlow II or benzodiazepine binding site, located on subdomain IIIA. Thus, albumin plays a relevant role in binding and transport functions and, therefore, on the pharmacokinetics of compounds.

Warfarin, a well-known anticoagulant drug commonly used in the prevention of thrombosis and thromboembolism, is pointed out as an albumin Sudlow I site marker. Thus, warfarin has been used in studies about displacement reactions for a variety of albumin-friendly drugs. To take higher advantage of the site marker role of warfarin, it is convenient to know precisely the binding parameters of warfarin itself with albumin. The aim of this work is to establish the thermodynamics of complete and reliable binding profiles of warfarin with two kinds of albumin, HSA and BSA, in environments close to the physiological ones.



Extracted from Sarsam et al, Metallomics (2011), 3, 152-161

EXPERIMENTAL

Isothermal titration calorimetry (ITC)

- Instrument: Microcal VP-ITC (MicroCal) titrator
- HSA and BSA solutions (cell): 0.01 - 0.02 mM in HEPES buffer (50 mM, pH=7.4).
- Warfarin solutions (syringe): 0.2 - 0.5 mM in HEPES buffer
- Temperature, 25±0.2˚C

Frontal analysis capillary electrophoresis (FA/CE)

- Instrument: CE Agilent Technologies capillary electrophoresis device equipped with a DAD detector
- Capillary: fused silica TSP (Polymicro Technologies), 48.5 cm total length, 40 cm effective length, 50 µm ID
- Separation solution and conditions : HEPES buffer, 25 ºC, 15 kV, positive polarity
- Injection: hydrodynamic, 0.5 psi, 80 s
- Albumin solutions: 12 µM for HSA and 57 µM for BSA in HEPES
- Warfarin solutions: from 8.5 to 950 µM in HEPES

$$r = \frac{[D_{bound}]}{[P_{total}]} = \sum_{i=1}^{m} n_i \frac{K_{bi}[D_{free}]}{1 + K_{bi}[D_{free}]}$$

Fluorescence quenching (FS)

- Instrument: Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies)
- Fluorimeter conditions: 1 cm path length quartz cuvette, synchronous mode at Δ=20nm; excitation wavelength 284 nm (HSA) or 287 nm (BSA)
- HSA and BSA solutions: 5 µM, 3 mL in HEPES (50 mM) or PBS (10 mM, I=150 mM, pH=7.4) buffers
- Warfarin solutions: 312.5 µM and 625 µM in HEPES or PBS buffers
- Temperature: 18, 25, 30, 37 or 42 ± 1ºC

Binding constant, K_b, was calculated using a new approach which includes the free drug concentration and involves a non-linear iteration. The enthalpy variation involved in each interaction has been calculated using the Van't Hoff equation.

$$[D_{total}] = \left(\frac{F_o - F}{F}\frac{1}{K_b}\right)^{1/n} + n[P_{total}]\left(\frac{F_o - F}{F_o}\right)$$

$$\ln K_b = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R}$$

RESULTS AND DISCUSSION

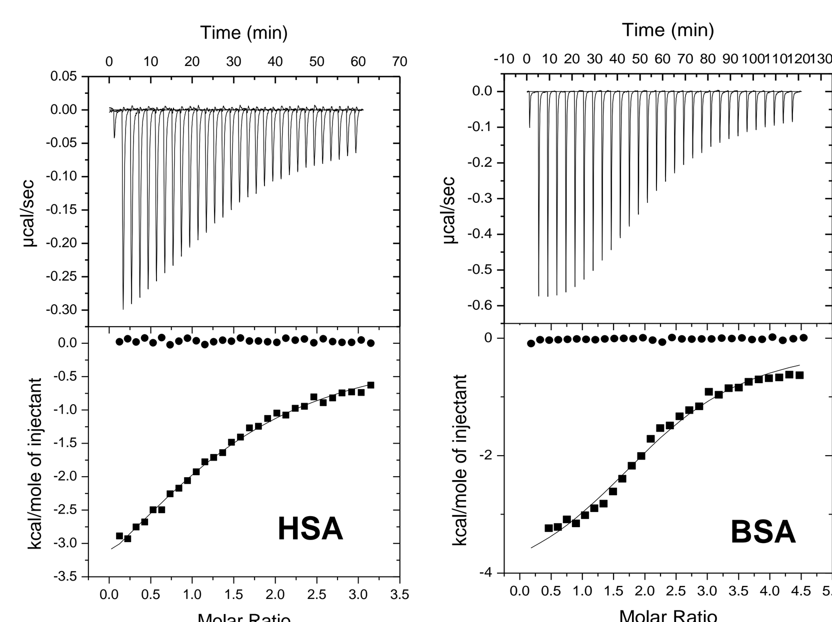
Measured warfarin-HSA and warfarin-BSA binding parameters

	n_1	K_{b1} (M)	ΔH_1 (kcal/mol)	n_2	K_{b2} (M)	Technique (buffer)
HSA	1.2±0.2	$(7\pm2)\times10^4$	-5.4±2.1	-	-	ITC(HEPES)
	0.92±0.01	$(4.2\pm0.5)\times10^4$	-5.6±1.0	-	-	FS (HEPES)
	2.0±0.1	$(3.7\pm0.4)\times10^4$	-	3.6±0.2	$(1.03\pm0.09)\times10^4$	FA/CE(HEPES)
	1.12±0.02	$(3.2\pm0.5)\times10^5$	-4.0±0.6			FS (PBS)
BSA	2.2±0.1	$(1.2\pm0.8)\times10^5$	-4.3±0.9	-	-	ITC (HEPES)
	0.96±0.01	$(0.8\pm0.1)\times10^5$	-3.7±0.1	-	-	FS (HEPES)
	1.62±0.08	$(1.1\pm0.2)\times10^5$	-	2.25±0.06	$(2.3\pm0.3)\times10^4$	FA/CE (HEPES)
	1.14±0.01	$(5\pm2)\times10^5$	-4.5±0.6			FS (PBS)

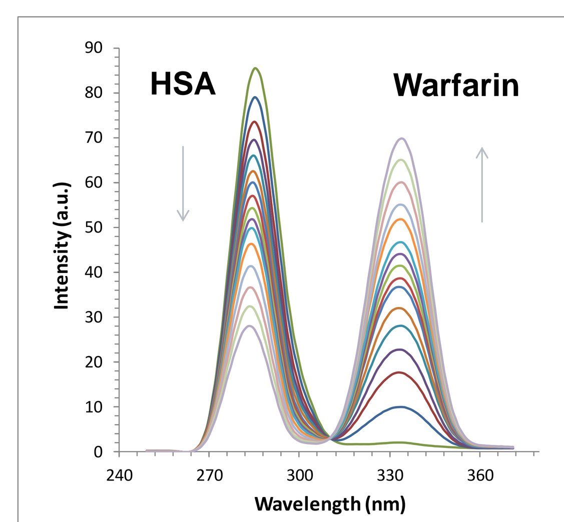
ITC measurements

There is a strong agreement between values obtained in HEPES buffer and those reported in literature for HSA. Then, ionic strength and buffer agent are irrelevant irrespective the involved thermodynamic quantities.

In the case of BSA, a high stoichiometry value has obtained in all cases. Titrations in HEPES buffer point out a binding constant somewhat higher than the literature value.

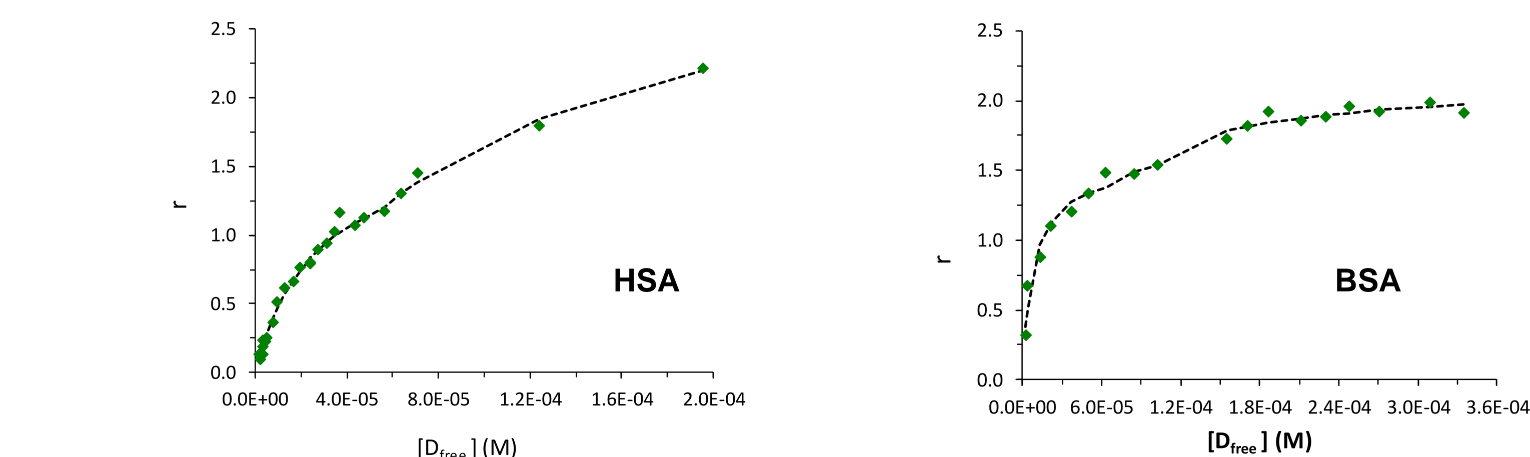


Fluorescence measurements



As shown in the figure, the BSA fluorescence is quenched when a growing volume of the warfarin solution is added to the system. Fluorescence quenching is related to the formation of the warfarin-albumin complex. The warfarin signal increases during the experiment due to the increase in the free-warfarin fraction. Both, warfarin-HSA and warfarin-BSA interactions in HEPES buffer show a stoichiometry close to the unity but a binding constant ten and five times lower, respectively, than those obtained in phosphate buffer. Then, the working solution composition has a significant role in this case.

FA/CE measurements



FA/CE measurements allow evaluating two different interaction steps between warfarin and HSA or BSA.

The high stoichiometry value for the first one and the similarity between both binding constants suggest some overlap between both steps in HEPES buffer. Thus, the lower differentiating ability of HEPES irrespective PBS buffer is pointed out.

The values obtained in HEPES buffer related to the first binding step are consistent with literature, whereas K_b for the second one is somewhat higher than those previously published.

Literature warfarin-HSA and warfarin-BSA binding parameters

n_1	K_{b1} (M)	ΔH_1 (kcal/mol)	n_2	K_{b2} (M)	Buffer	Technique
HSA						
0.85	4.9×10^4	-4.83	-	-	MOPS (I=100 mM)	ITC[1]
0.98	6.6×10^4	-6.24	-	-	PBS (I=100 mM)	ITC[1]
1.0	1.4×10^6	-	-	-	PBS (5 mM)	FS[3]
0.7	8.6×10^5	-	-	-	PBS (5 mM)+ NaCl (50 mM)	FS[3]
0.5	4.5×10^5	-	-	-	PBS (5 mM)+ NaCl (200 mM)	FS[3]
0.88	3.59×10^5	-1.2	-	-	PBS (10 mM) +NaCl (0.9%)	FS[4]
-	2.4×10^5	-	-	-	PBS (9.5 mM) + NaCl (137 mM)	FS[5,6]
-	2.3×10^3	-	-	-	PBS (50 mM) + NaCl (100 mM)	FS[7]
-	2.8×10^5	-	-	1.4×10^4	PBS (67 mM)	FS[8]
1.38	3.30×10^5	-	-	-	PBS (67 mM)	FS[9]
1.5	1.1×10^5	-	2.9	7.7×10^3	PBS (67 mM)	CE/FA[10]
1.4	1.2×10^5	-	2.8	1.2×10^4	PBS (67 mM)	CE/FA[10]
1.0	3×10^5	-	2.8	7.4×10^3	PBS (67 mM)	CE/FA[11]
2.3	4.0×10^4	-	2.8	3.5×10^2	PBS (66.7 mM)	CE/FA[12]
-	1.67×10^5	-5.3	-	4.83×10^4	PBS (66 mM)	ED[14]
1.0	3.04×10^5	-	2.0	2.92×10^4	PBS (67 mM)	ED[15]
BSA						
2.5	4.76×10^4	-	-	-	PBS (50 mM)	ITC[2]
-	8.7×10^4	-	-	-	PBS	FS[5]
-	2.9×10^4	-	-	-	PBS (50 mM)+NaCl (100 mM)	FS[7]
1.2	1.8×10^5	-	2.5	5.6×10^3	PBS (67 mM)	CE/FA[11]
1.09	2.4×10^5	-	1.92	4.1×10^3	PBS (67 mM)	CE/FA[13]
1.0	2.65×10^5	-	2.0	2.02×10^4	PBS (67 mM)	ED[15]

ITC meaurements: [1] Photochem. Photobiol 82 (2006) 1365-1369, [2] Magn. Reson. Chem. 43 (2005) 463-470

FS measurements: [3] J. Phys. Chem. B 118 (2014) 26-36, [4] Mol. Pharmacol. 21 (1981) 100-107, [5] J. Luminiscence 154 (2014) 767-773, [6] J. Luminiscence 142 (2013) 122-127, [7] Biochim. Biophys. Acta 1721 (2005) 164-173, [8] Protein Sci. 9 (2000) 1455-1465, [9] J. Photochem. Photobiol. 95 (2009) 189-195

FA/CE measurements: [10] Anal. Chem. (2003) 207-214, [11] J. Chromatogr. A 777 (1997) 311-328, [12] J. Chromatogr. A 1238 (2012) 146-151, [13] J. Pharm. Biomed. Anal. 53 (2010) 1288-1297

ED measurements: [14] Mol. Pharmacol. 27 (1985) 263-270, [15] Pharm. Research 14 (1997) 1607-1612

CONCLUSIONS

- Thermodynamics of warfarin-albumin interactions can be successfully evaluated by means of several analytical techniques (ITC, FS or FA/CE).
- The first interaction binding values are strongly consistent within the selected techniques whatever the buffer used (HEPES or PBS). However, in case of HSA, values in HEPES buffer are somewhat lower than those derived from PBS buffer.
- The drug/protein ratio is the unity when measured by FS but results from ITC lead to a binding stoichiometry slightly higher than the unity for HSA and twice than expected for BSA. These last values can be attributed to some contribution of high order interactions clearly shown by FA/CE, the only approach able to detect two distinct binding episodes for studied systems.
- FS can be strongly recommended when only first binding event should be considered, whereas ITC or FA/CE should be selected for consideration of global binding process. ITC allows, in addition, an accurate evaluation of thermodynamic binding parameters.