Molecular interactions between warfarin and human (HSA) or bovine (BSA) serum albumin evaluated by Isothermal Titration Calorimetry (ITC), Fluorescence Spectrometry (FS) and Frontal Analysis Capillary Electrophoresis (FA/CE)

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ABSTRACT

Interaction thermodynamics between warfarin, a very popular anticoagulant, and Sudlow I binding site of human (HSA) or bovine (BSA) serum albumin have been examined in strictly controlled experimental conditions (HEPES buffer 50 mM, pH 7.4 and 25 ºC) by means of isothermal titration calorimetry (ITC), fluorescence spectrometry (FS) and frontal analysis capillary electrophoresis (FA/CE). Each technique is based on measurements of a different property of the biochemical system, and then the results allow a critical discussion about the suitability of each approach to estimate the drug-protein binding parameters. The strongest interaction step is properly evaluated by the three assayed approaches being the derived binding constants strongly consistent: from $4x10^4$ to $7x10^4$ for HSA and from $0.8x10^5$ to $1.2x10^5$ for BSA. Binding enthalpy variations also show consistent results: -5.4 and -5.6 Kcal mol^{-1} for HSA and -4.3 and -3.7 Kcal mol^{-1} for BSA, as measured by ITC and FS, respectively. Further high order interaction events for both albumins are detected only by FA/CE.

Keywords:

Warfarin-serum albumin interactions; isothermal titration calorimetry; fluorescence spectrometry; frontal analysis capillary electrophoresis

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1. Introduction

Albumin, the most abundant protein in plasma and serum, is a water-soluble macromolecule, which permits to maintain the plasma oncotic pressure and modulate the fluid distribution among body compartments. It shows also considerable buffering, antioxidant and pseudo-enzymatic abilities. Native albumin is built up from three homologous domains (I, II and III), each one with two distinct subdomains, named A and B. Drugs and other compounds bind mainly to 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 [1,2]. Thus, albumin plays a relevant role on the pharmacokinetics of drugs. Albumin molecular structure strongly depends on its origin and only about 70% of the macromolecule is a common moiety for any kind of albumin. Thus, it has been noticed about 25-30% of variability in amino acid sequences according to the albumin origin, human (HSA), bovine (BSA) or others [2,3]. For example, HSA has only one tryptophan residue (Trp-214) while BSA contains two of them (Trp-134 and Trp-213), being the single Trp-214 of HSA located in a similar microenvironment as the Trp-213 of BSA. BSA Trp-134 is in a rather superficial site and shows a lesser hydrophobic character. Consequently, rather different binding activities of both albumins can be expected. Although HSA is usually preferred in pharmacological studies, BSA is often selected because of veterinary conveniences and easier availability. In recent and comprehensive reviews on the nature of HSA Sudlow I [4], Abou-Zied states that it is able to bind a variety of ligands by adapting its binding pockets. Moreover, the site binding ability strongly depends on the number of trapped water molecules, which increases with the unfolding and refolding HSA sample past. Then, binding values between a particular drug and HSA Sudlow I site significantly depend on the quality of the protein sample. Literature do not show details about the BSA Sudlow I behavior, but it seems plausible to expect similar binding ability constrictions.

Warfarin is a well-known anticoagulant drug commonly used in the prevention of thrombosis and thromboembolism. It helps the blood to flow freely around the body stopping the clots formation and playing an essential role on the drug pharmacokinetics [4]. From a physicochemical point of view, warfarin shows moderate acidity (pK_a = 5.0) [5], significant lipophilicity (log $P_{o/w}$= 3.2, log $D_{pH=7.4}$ = 0.9) [6,7] and low aqueous intrinsic solubility (S_0 = 5.3 mg L^{-1}) [8]. Many studies point out warfarin as an albumin Sudlow I site marker [4,9], despite Dockal et al. [10] indicate the indispensable structural contributions of subdomain IIB and

domain I. Warfarin has been used in studies about displacement reactions for a variety of albumin-friendly drugs. Nevertheless, to interpret rightly the results, it is convenient to know precisely the binding parameters of warfarin itself with albumin. Literature values about stoichiometry and energetics of warfarin interactions with HSA or BSA at physiological pH are summarized in Table 1.

The aim of this work is to establish reliable binding profiles of warfarin with HSA and BSA, in environments close to the physiological ones. Several common techniques and strictly controlled experimental conditions have been chosen to contrast efficiently the binding values derived from each approach. Thus, isothermal titration calorimetry (ITC), fluorescence spectrometry (FS), and frontal analysis capillary electrophoresis (FA/CE) have been selected. As well known, ITC is able to measure directly the energy and it is, in some sense, the reference approach. By contrast, FS is focused on the quenching effect of drug on the protein intrinsic fluorescence whereas FA/CE is based in the estimation of the bonded drug and total protein concentrations ratio when electrophoretic mobility of the protein equals that of the drug-protein complex. Thus, the two last approaches allow the determination of stoichiometry and binding constant as significant interaction parameters. Selected techniques show different sensitivity limits and, then, not all of them are able to measure properly hypothetical successive binding steps [11,12]. Consequently, a critical comparison about the binding parameters achieved by the tested techniques on a relatively simple biological interaction (just a well-known acidic drug binding, mainly, the Sudlow I site of native albumin), using albumin of two biological origins (HSA and BSA), in strictly controlled conditions (HEPES buffer, pH=7.4, I=50 mM, T=25 ºC) is performed. Results and conclusions of this work should facilitate further studies in which warfarin is involved, such as its displacement by other drugs with higher affinity with the albumin.

2. Experimental

2.1 Chemicals

Sigma-Aldrich (St Louis, MO, USA) human serum albumin (HSA) (99%) and bovine serum albumin (BSA) (>99%) were used after spectrophotometric verification of purity. Sigma-Aldrich warfarin (>98%) and (N-2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) (>99.5%) were used as received. To adjust the

working pH, 0.5 M NaOH (Titrisol, Merck, Darmstadt, Germany) or 0.5 M HCl (Titrisol, Merck) were employed. Na_2HPO_4 (>99%) and KH_2PO_4, KCl, NaCl (>99.5%) from Merck are used too. To prepare the buffer and the sample solutions, and to standardize and clean the microcalorimeter, water purified by a Milli-Q-plus system with a resistance higher than 18 MΩ·cm was used.

A 0.2 M HEPES buffer solution (pH 7.4 and ionic strength 50 mM) previously neutralized with NaOH was prepared. An adequate amount of HCl was added to an aliquot of the previous solution to get the chosen pH value and lastly diluted to a final concentration of 50 mM. Working in this way the buffer concentration equals its ionic strength, which is calculated assuming that zwitterions do not contribute to the ionic strength of the solution [12]. PBS buffer solution (pH 7.4, 140 mM of chloride and 150 mM ionic strength) was prepared by mixing 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl and 2.7 mM KCl, and final correction to get the selected pH. The ionic strength of the HEPES buffer was set at 50 mM to avoid the Joule effect in the EC measurements, that occurred over 100 mM. This condition was kept constant when using the other two analytical techniques to better compare the results. In the case of the measurements in PBS solutions by FS, it was used a concentration of phosphates of 10 mM (150 mM ionic strength) because under these conditions the FS measurements are performed closer to the *in vivo* medium.

2.2 ITC titrations

2.2.1 Instruments

A Microcal VP-ITC (MicroCal, LLC, Northampton, MA, USA) titrator was used. Working solutions were degassed by means of a Thermovac (MicroCal, LLC) vacuum degasser. The instrumental response was checked by means of the chelation reaction of Ca^{2+} with EDTA [13]. 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) was used for pH measurements. The electrode system was calibrated with ordinary aqueous buffers of pH 4.01 and 7.00.

82 2.2.2 Procedure

83 Albumin and warfarin were solved with HEPES buffer solution. Both HSA and BSA solutions were in the 0.01
84 to 0.02 mM range, and the concentration of drug solutions varied from 0.2 to 0.5 mM. Prior to their use, all
85 solutions were degassed for a period of 5 min at 24 ºC. Titrations were performed at 25.0±0.2 ˚C. The power
86 reference was 10 µcal s^{-1} and the stirring rate was 290 rpm to ensure rapid mixing. The injection volume was
87 8 µL and the interval between injections was 240 s to warrant the equilibrium in each titration point. Each
88 titration involved 29 independent titrant additions. The syringe was filled with the warfarin solution whereas
89 the albumin was in the cell. Background titrations were performed according to: a) identical titrant solution
90 with the cell filled just with the buffer and b) successive buffer additions to the albumin solutions. These
91 measurements allow the determination of the background heat to be subtracted to the main titrations.
92 Moreover, the dilution heat of the drug-protein complex was evaluated by successive buffer additions to the
93 complex solutions. Each assay was repeated several times.

94

95 2.2.3 Calculations

96 Origin 7.0 software supplied by Microcal was used for data treatment. Experimental data were collected
97 automatically and analyzed to get the interaction stoichiometry, *n*, and the binding quantities associated to
98 the interaction event, ΔH and K_b. The suitable adjusting model (one, two or sequential binding sites) should
99 be introduced into the software.

100

101 2.3 Fluorimetric measurements

102

103 2.3.1 Instruments

104 Fluorescence measurements were performed on a Cary Eclipse Fluorescence Spectrophotometer from
105 Agilent Technologies (Sta Clara, CA, USA) using a 1 cm path length quartz cuvette, a medium scan speed, slit
106 widths with a nominal band-pass of 5 nm for both excitation and emission, and a Savitzky-Golay smooth of
107 19. The mentioned Crison micro-pH 2002 pH meter was used for pH measurements. A magnetic heater stirrer
108 (Agimatic-N, JP Selecta, Abrera, Spain) was used too.

109

110 2.3.2 Procedure

111 First, the emission spectra of warfarin (30 μM in HEPES or PBS buffer, excitation wavelength: 315 nm), HSA
112 (5 μM in HEPES or PBS buffer, excitation wavelength: 284 nm) and BSA (5 μM in HEPES or PBS buffer,
113 excitation wavelength: 287 nm) were recorded. Warfarin, HSA and BSA showed maximum emission signals
114 at 392 nm, 344 nm, and 346, respectively. To avoid warfarin interference in HSA and BSA fluorescence
115 quenching evaluation, measurements were done using the Synchronous mode at $\Delta\lambda$=20 nm (λ_{em}=284 nm for
116 HSA and 287 nm for BSA). Then, the linear range of HSA and BSA fluorescence was evaluated. In working
117 conditions, the linear range for both albumins was between 0.5 and 8 μM. Therefore, 5 μM HSA or BSA was
118 the chosen concentration.
119 Quenching studies were carried out by titration. The albumin (5 μM, 3 mL) was placed in the cuvette and the
120 solution was taken up to the temperature under study (18, 25, 30 or 37 ± 1 ºC) using a water bath and a
121 magnetic stirrer. The initial fluorescence synchronous spectrum was recorded. Nine successive additions of
122 warfarin (312.5 μM in HEPES or PBS buffer, 7 μL) and five new additions of warfarin (625 μM in HEPES or PBS
123 buffer; 5, 8, 11, 14 and 17 μL) were done. After each addition, the solution in the cuvette was stirred (3 min,
124 900 rpm) to allow the temperature equilibration and the reaction happening. Finally, the fluorescence was
125 measured in the synchronous mode.

126

127 2.3.3 Calculations

128 The experimental data were exported from the Cary Eclipse Scan Application software to an Excel sheet
129 (Microsoft, Redmond, WA, USA), and the fluorescence data were corrected for the dilutions carried out along
130 the titration.
131 Fluorescence measurements at various temperatures (from 18 to 37 ºC) were performed and the derived
132 Stern-Volmer constant values (K_{SV}) were calculated by means of Eq. 1

133 $$\frac{F_0}{F} = 1 + K_{\mathrm{SV}}[D_{tota}\,] \qquad \text{[Eq. 1]}$$

134 where F_0 is the initial albumin fluorescence, F is the albumin fluorescence after the quencher addition, and
135 [D_{total}] is the total drug concentration.

The interaction stoichiometry, n, and the binding constant, K_b, were calculated using two different approaches. The first one uses a linear equation fitting, Eq. 2.

$$\log \frac{F_o - F}{F} = \log K_b + n \log[D_{total}] \qquad \text{[Eq. 2]}$$

The second approach involves a non-linear equation (Eq. 3) which includes the free drug concentration (in the second term of the right side) and the total protein, P_{total} , concentration

$$[D_{total}] = \left(\frac{F_o - F}{F} \frac{1}{K_b} \right)^{1/n} + n [P_{total}] \left(\frac{F_o - F}{F_o} \right) \qquad \text{[Eq. 3]}$$

The Excel solver tool allows an easy estimation of n and K_b quantities. The enthalpy variation, ΔH^0, was calculated using the van't Hoff equation, Eq. 4, where ΔS^0 stand for the entropy variation involved in the binding event

$$\ln K_b = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} \qquad \text{[Eq. 4]}$$

2.4 FA/CE measurements

2.4.1 Instruments

An Agilent capillary electrophoresis equipped with a diode array detector operating at 214 nm was used. The measurements were performed at 25.0±0.1˚C on an uncoated fused-silica capillary (50 cm effective length x 50 µm internal diameter) from Polymicro Technologies (Phoenix, AZ, USA). The working conditions included the application of a 15 kV voltage and positive polarity. For pH measurements, the Crison micro-pH 2002 pH meter described in Section 2.2.1 was used.

2.4.2. 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.

HEPES buffer was used as the separation solution. Calibration curve was built from free warfarin solutions (2-250 µM for HSA and 8-530 µM for BSA). Constant albumin concentration (12 µM for HSA and 57 µM for BSA) and variable warfarin concentration (6-240 µM for HSA and 21-460 µM for BSA) solutions, all of them in HEPES buffer, were also prepared. To obtain the "plateau" signal the sample was injected hydrodynamically at 0.5 psi for 80 s.

2.4.3 Calculations

Experimental data were exported from the Agilent CE Chem Station software to an Excel sheet to record the "plateau" height and were treated according to Eq. 5

$$r = \frac{[D_{bound}]}{[P_{total}]} = \sum_{i=1}^{m} n_i \frac{K_{bi}[D_{free}]}{1+K_{bi}[D_{free}]} \quad \text{[Eq. 5]}$$

where [D_{bound}] and [D_{free}] stand for the concentration of the bound 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. The symbol *m* denotes the total number of different binding sites by a particular drug-protein system and *r* stands for the ratio between the concentrations of bound drug and total protein [11]. Interaction parameters have been calculated by direct adjust of experimental points to Eq. 5 through Excel software using a supplementary optimization Excel Macro [14]. Nevertheless, the Scatchard and Klotz approaches have been also considered because their linearized models facilitate an easy view of the successive interactions and also the dispersion of the experimental data irrespective to the considered model [11].

3. Results and discussion

Table 1 compiles literature values for warfarin interactions with both albumins, HSA and BSA, derived from a variety of approaches performed in similar experimental conditions. The whole pool of values shows that

some works report just one drug-protein binding event but some others split the global interaction into two consecutive steps. The first group involves the measurements made by ITC or FS, that is, by analytical tools able to measure efficiently from moderate to high affinity events. By contrast, the works included in the second group were performed by any of two separation techniques, CE/FA or equilibrium dialysis (ED), which are able to evaluate properly weak and moderate binding interactions [11]. The only exception is reported by Dockal et al. [10] in a FS study made with HSA and several of its recombinant fragments, which leads to a two-steps interaction without any indication about the irrespective binding stoichiometries. The mentioned technique-dependent sensitivity ranges were experimentally verified for ITC and FA/CE in a previous study about the interactions of some anti-inflammatory drugs with albumin, in which Sudlow II site is directly involved. It was concluded that a suitable combination of these complementary techniques could lead to reliable binding profiles [12]. Table 1 shows also that the stronger binding event (or the unique one) implies, in many instances, a binding constant value around 10^5 for both albumins. Reported stoichiometry is close to the unity with the exception of the ITC results for warfarin-BSA interaction, which is much higher [9]. In all two-step binding reports, the differences between the consecutive binding constant values are between one and two orders of magnitude and the calculated stoichiometry for the second event ranges between 2 and 3. These data point out a relatively strong specific interaction of warfarin with both albumins and additional interactions associated to weaker binding episodes.

Table 1 shows also that almost all literature studies were done in phosphate buffer (PBS). However, phosphate, as well as citrate, borate and succinate, have some disadvantages for studies of biological or complex systems. Thus, PBS has a poor buffering capacity above pH 7.5 since its dissociation constant is about 6.8 at plasmatic ionic strength and, in addition, it is an active participant in many biochemical processes, inhibiting the enzymes catalytic role for instance. PBS also demonstrate complexing capabilities with polyvalent cations and can inhibit several metal ion-dependent biochemical reactions. On the contrary, PIPES, HEPES, MES and MOPSO, are adequate substitutes of Tris or PBS showing better chemical behavior than other zwitterionic buffers and they have been, recently, strongly recommended [15]. Thus, despite some warnings about possible interferences of HEPES with ligands binding HSA (Biacore Symposium 2002, Chicago, Illinois, 1-17), it has been selected in present work as a suitable option.

3.1 In-house experimental results vs. literature data

3.1.1 ITC measurements

To get the best working conditions, several titrations with different concentration ratios between warfarin and HSA or BSA have been tested. The poor aqueous solubility of warfarin has compelled to select much diluted solutions being the best titration curves those with an initial drug-albumin ratio about 17 for HSA titrations and 20 for BSA titrations, shown in Figure 1. In both instances, calorimetric curves show an enthalpy gap between the end of the jump and the blank signal, which suggests additional warfarin-albumin interactions. Derived binding parameters are gathered in Table 2.

Regarding ITC literature data for warfarin-HSA interaction, it should be pointed out the strong agreement between values obtained in phosphate and MOPS buffers both at the same ionic strength (100 mM) [16],and that derived in this work from HEPES buffer (50 mM), see Tables 1 and 2. They show that ionic strength and buffer agents are irrelevant irrespective the involved thermodynamic binding quantities (notice the buffer deprotonation molar enthalpy values: $\Delta H_{diss(H2PO4-)}$= 3.6 kJ mol^{-1}, $\Delta H_{diss(MOPS)}$= 21.1 kJ mol^{-1}and $\Delta H_{diss(HEPES)}$= 20.4 kJ mol^{-1} [17]). Thus, it is concluded that the binding reaction is not coupled with gain or release of protons by HSA or warfarin [18]. Therefore, the measured enthalpy variation should be very close to the true drug-albumin binding enthalpy since no additional side reactions in the working cell are foreseeable. However, data derived from Tris (100 mM) solutions show a higher binding constant, which could be attributed to the very low buffer capacity of Tris at pH 7.13 and, then, to some heat contribution of buffer itself (p$K_{a(TrisH+)}$= 8.07; $\Delta H_{diss(TrisH+)}$= 47.45 kJ mol^{-1} [17]). In any case, reported binding constant [16] is consistent with most values compiled in Table 1.

Only one literature reference is devoted to ITC determination of warfarin-BSA interaction but no associated enthalpy datum is stated. Surprisingly, it reports a very high stoichiometry value. The authors claim that the raw data seem to point out that the drug binds to albumin at more than one binding site and conclude that warfarin-BSA is not a good model system for protein-ligand interactions [9]. Titrations made in this work also point out high stoichiometry and a binding constant somewhat higher than the reported value.

244

245 3.1.2 FS measurements

246 The in-house fluorescence measurements were performed at various temperatures and fitted to Stern-
247 Volmer relationships in order to estimate the K_{SV} values (Eq. 1). The results, gathered in Table 3, allow the
248 verification of the static character of the warfarin quenching effect on albumin fluorescence [19]. Therefore,
249 interaction parameters can be calculated by means of the most common approach, Eq. 2, despite it involves
250 the statement that free and total drug concentrations are equivalent. To overcome this simplifying
251 assumption and to get a more precise calculation tool, a new expression has been derived in this work (Eq.
252 3, Appendix A). It also assumes the protein as the only fluorophore agent in the biological system but it
253 embodies the free drug concentration. Experimental data for interactions of warfarin with both albumins,
254 HSA and BSA, were fitted to Eq. 3 and the results are shown in Table 2.
255 Literature binding parameters for warfarin-HSA interaction were measured in PBS buffer at various ionic
256 strength levels. K_b values derived from solutions with high NaCl contents are about $3x10^5$ and reported
257 stoichiometry ranges between 0.5 and 1.4, see Table 1. It should be noticed the decrease in K_b and n values
258 with the increase of the NaCl content, as stated in the systematic Bolel's study [20]. This behavior can be
259 explained since chloride anions present in the solution compete with warfarin resulting in a decrease in
260 estimated warfarin-albumin binding constant [21,22]. Despite NaCl addition to buffer solutions is not
261 mentioned in a couple of literature reports [10,23], the buffer preparation itself requires non-negligible HCl
262 amount if the very common Na_2HPO_4 is the main chemical used. Unfortunately, detailed buffer preparation
263 is not described in the original manuscripts. Results from this work (HEPES buffer 50 mM and chloride about
264 30 mM) show a stoichiometry close to the unity but a binding constant which is almost one order of
265 magnitude lower than most values previously published. This result could be attributed to the used fitting
266 model (Eq. 3) since slightly higher binding quantities were obtained when the same experimental data pool
267 was fitted to the common Eq. 2 (n=1.0; K_b=$8.2x10^4$). The recalculated K_b remains, however, somewhat lower
268 than most values displayed in Table 1 showing that used background solutions must be considered in
269 comparative studies. To verify again the working solution effect, Table 2 also includes the in-house results
270 obtained in PBS, which agree with those previously published and shown in Table 1. In any case, final results

271 achieved in this work show an equimolar drug-protein interaction and a binding constant strongly consistent
272 with the value attained by ITC in identical working conditions.
273 Literature data for warfarin-BSA interaction show a similar behavior. Thus K_b value evaluated from FS
274 measurements in buffered solutions with a high content of NaCl is lower than the one obtained in plain
275 buffer, see Table 1. Our own measurements lead to a binding constant value consistent with that reported
276 by Poor [2] and, such as in warfarin-HSA instance, when Eq. 2 was used slightly higher binding parameters
277 were obtained (n=1.1; K_b=3.0x10^5). To evaluate again the combined effect of buffer and chloride ions, new
278 measurements in PBS buffer were performed. As expected calculated binding constant is about five times
279 the one obtained in HEPES buffer, revealing again the significant role of the working solution composition,
280 see Table 2.
281 Finally, the fit of K_b values at various temperatures to the Vant'Hoff equation (Eq. 4) (slope = 2819, intercept
282 = 1.22, n = 3 and R^2 = 0.966 for HSA; and slope = 1621, intercept = 5.92 , n = 4 and R^2 = 0.982 for BSA) allows
283 the estimation of the molar enthalpy variation involved in each interaction. Results are gathered in Table 2,
284 showing the consistency achieved from both ITC and FS approaches.
285
286 3.1.3 FA/CE measurements
287 Table 1 shows two distinct binding steps for warfarin-albumin interaction when measured by any separation
288 technique. Working temperature was 25 or 37 ºC but no significant differences in final results were reported.
289 Warfarin-HSA interaction plot measured in HEPES buffer is shown in Figure 2A. It suggests two successive
290 steps that were verified by means of the Scatchard and Klotz linear approaches. Experimental data were
291 adjusted to Eq. 5 and derived parameters indicate very similar binding constants for both binding episodes,
292 see Table 2. The high stoichiometry value for the first one and the similarity between both binding constants
293 suggest some overlap between both steps in working conditions. Thus, results show the lower differentiating
294 ability of HEPES irrespective PBS buffer (see Table 1) pointing out, however, the presence of higher order
295 binding events, which are common when a charged species tents to interact with a poly-charged protein [12].
296 Figure 2B depicted the binding curve for warfarin-BSA. Data were treated in the same way explained for HSA
297 and binding parameters of successive episodes were calculated and included in Table 2. Values related to the

298 first binding step are consistent with literature whereas K_b for the second one is somewhat higher than those
299 previously published, see Tables 1 and 2.
300
301 3.2 Some remarks about the binding results
302 Despite the diversity among the evaluated quantities by the used analytical tools (ITC, FS and FA/CE), binding
303 constant values determined in HEPES buffer for warfarin-albumin first interaction event is strongly consistent
304 for both tested albumins, HSA or BSA. Nevertheless, the drug/protein ratio is the unity when measured by FS
305 whereas results from ITC lead to a binding stoichiometry slightly higher than the unity for HSA and twice than
306 expected for BSA. These last values can be attributed to some contribution of high order interactions clearly
307 shown by FA/CE, the only approach able to detect two distinct binding episodes for studied systems. The
308 same reason could explain the stoichiometry higher than the unity for the first binding events measured by
309 FA/CE. To evaluate properly these results, it should be taken into account that binding affinity values
310 obtained by FS just took in consideration the location of fluorophores since such technique simply measures
311 the local changes around them whereas the calorimetric approach considers the overall global changes [22].
312 CE/FA also measures the global interaction because the mobility of the entire species is involved in
313 calculations. Nevertheless, K_b values evaluated by the three selected approaches are consistent (Table 2) and
314 all tested techniques should be considered suitable tools to solve the problem in hand.
315 In summary, at least two distinct binding events are involved in studied interactions. First episode binding
316 constants are robust being the value for warfarin-HSA about one half order of magnitude lower than that of
317 warfarin-BSA (Table 2). Higher order binding events involve similar energy in both instances.
318 Since this work is performed in HEPES buffer and most literature binding values have been derived from PBS
319 buffered solutions, a comment about the buffer effect on binding parameter values seems to be appropriate.
320 Then, regarding warfarin-HSA first interaction, ITC results obtained from both buffered solutions agree,
321 whereas binding values from PBS solutions are about one order of magnitude higher than those obtained
322 from HEPES buffer when FS or FA/CE were used. By contrast, values obtained for warfarin-BSA interaction in
323 both buffered solutions are consistent. For both albumins, PBS buffer allows better resolution between

successive binding events than HEPES buffer. Thus, background solution significantly affects the binding parameters and should be considered in further biological studies.

4. Conclusions

Thermodynamics of warfarin-albumin interactions in strictly controlled conditions (HEPES buffer, pH 7.4 and ionic strength 50 mM) can be successfully evaluated by means of several analytical techniques (ITC, FS or FA/CE). Experimental conditions such as pH, buffering agent and contents of chloride ions in working solutions should be fixed to get reliable binding values. HEPES buffer is considered free of common side reactions often present in biochemical systems and lead to reliable binding parameters. Thus, first interaction step binding values obtained by means of the selected techniques are strongly consistent and, in case of HSA, somewhat lower than those derived from PBS buffer. Warfarin-BSA system shows similar binding parameters when measured from both buffers, HEPES and PBS.

Regarding to 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.

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Conflict of interest

The authors declare no competing financial interest.

Figure captions

Figure 1: ITC titration curves of serum albumin (A: HSA; B: BSA) with warfarin

Figure 2 : FA/CE binding curves for warfarin-albumin system (A: HSA; B: BSA)

Table 1. Literature data for interactions between warfarin and human (HSA) or bovine (BSA) serum albumin

n_1	K_{b1} (M)	ΔH_1(Kcal/mol)	n_2	K_{b2} (M)	*pH*	*T* (˚C)	Buffer (concentration or ionic strength, *I*)	Experimental technique*	Reference
HSA									
0.97	$1.63x10^5$	-3.06	-	-	7.13	25	Tris (*I*=100 mM)	ITC	[16]
0.85	$4.9x10^4$	-4.83	-	-	7.13	25	MOPS (*I*=100 mM)	ITC	[16]
0.98	$6.6x10^4$	-6.24	-	-	7.13	25	Phosphate (*I*=100 mM)	ITC	[16]
1	$1.4x10^6$	-	-	-	7.3	25	Phosphate (5 mM)	FS	[20]
0.7	$8.6x10^5$	-	-	-	7.3	25	Phosphate (5 mM)+ NaCl (50 mM)	FS	[20]
0.5	$4.5x10^5$	-	-	-	7.3	25	Phosphate (5 mM)+ NaCl (200 mM)	FS	[20]
0.88	$3.59x10^5$	-1.2	-	-	-	27	Phosphate (10 mM) +NaCl (0.9%)	FS	[24]
-	$2.4x10^5$	--	-	-	7.4	25	Phosphate (9.5 mM) + NaCl (137 mM)	FS	[2,25]
-	$2.3x10^3$	-	-	-	-	25	Phosphate (50 mM) + NaCl (100 mM)	FS	[26]
-	$2.8x10^5$	-	-	$1.4x10^4$	7.4	25	Phosphate (67 mM)	FS	[10]
1.38	$3.30x10^5$	-	-	-	7.4	25	Phosphate (67 mM)	FS	[23]
1.5	$1.1x10^5$	-	2.9	$7.7x10^3$	7.4	37	Phosphate (67 mM)	CE/FA	[27]
1.4	$1.2x10^5$	-	2.8	$1.2x10^4$	7.4	37	Phosphate (67 mM)	CE/FA	[28]
1.0	$3x10^5$	-	2.8	$7.4x10^3$	7.4	27	Phosphate (67 mM)	CE/FA	[29]
2.3	$4.0x10^4$	-	2.8	$3.5x10^2$	7.4	-	Phosphate (66.7 mM)	CE/FA***	[30]
-	$1.67x10^5$	-5.3**	-	$4.83x10^4$	7.4	37	Phosphate (66 mM)	ED	[31]
1	$3.04x10^5$	-	2	$2.92x10^4$	7.4	25	Phosphate (67 mM)	ED	[3]
-	$2.14x10^5$	-	-	-	-	-	-	Various	[32]
1	$3.4x10^5$	-	-	-	7.4	-	-	-	[21]
-	$2.4x10^5$	-	-	-	-	-	-	-	[33]
BSA									
2.5	$4.76x10^4$	-	-	-	7.4	25	Phosphate (50 mM)	ITC	[9]
-	$8.7x10^4$	-	-	-	7.4	-	Phosphate	FS	[2]
-	$2.9x10^4$	-	-	-	7.4	25	Phosphate (50 mM)+NaCl (100 mM)	FS	[26]
1.2	$1.8x10^5$	-	2.5	$5.6x10^3$	7.4	-	Phosphate (67 mM)	CE/FA	[29]
1.09	$2.4x10^5$	-	1.92	$4.1x10^3$	7.4	-	Phosphate (67 mM)	CE/FA	[11]
1	$2.65x10^5$	-	2	$2.02x10^4$	7.4	25	Phosphate (67 mM)	ED	[3]

*Acronyms. ED: Equilibrium Dialysis; FS: Fluorescence Spectroscopy; ITC: Isothermal Titration Calorimetry; CE/FA: Capillary Electrophoresis Frontal Analysis;
** Value associated to the global interaction (two steps); *** Coated capillary with polyelectrolyte multilayers

Table 2. Binding parameters for warfarin and human (HSA) or bovine (BSA) serum albumin interactions obtained in this work. Experimental conditions: Buffer, HEPES or PBS (phosphate), pH 7.4; Temperature, 25 ºC.

Buffer	n_1	K_{b1} (M)	ΔH_1 (Kcal/mol)	n_2	K_{b2} (M)	Experimental technique (working molar ratio range)
HSA						
HEPES 50 mM *I*=50mM	1.21±0.18	(7.08±2.00)x10^4	-5.4±2.1	-	-	**ITC** (0.1-3.5)
HEPES 50 mM *I*=50mM	0.92±0.01	(4.21±0.51)x10^4	-5.6±1.0	-	-	**FS** (0.1-5.0)
HEPES 50 mM *I*=50mM	1.95±0.10	(3.7±0.4)x10^4	-	3.6±0.2	(1.03±0.09)x10^4	**FA/CE** (Step 1: 0.3-4.5; Step 2: 5.0- 13.2)
PBS 10 mM *I*=150mM	1.12±0.02	(3.18±1.5)x10^5	-4.0±0.6	-	-	**FS** (0.3-14)
BSA						
HEPES 50 mM *I*=50mM	2.20±0.13	(1.2±0.8)x10^5	-4.3±0.9	-	-	**ITC** (0.1-4.5)
HEPES 50 mM *I*=50mM	0.96±0.01	(0.8±0.1)x10^5	-3.7±0.1	-	-	**FS** (0.1-5.0)
HEPES 50 mM *I*=50mM	1.62±0.08	(1.08±0.22)x10^5	-	2.25±0.06	(2.28±0.31)x10^4	**FA/CE** (Step 1: 0.4-3.0; Step 2: 3.0-8.0)
PBS 10 mM *I*=150mM	1.14±0.01	(5.1±1.6)x10^5	-4.5±0.6	-	-	**FS** (0.3-12.5)

370 **Table 3**. Stern-Volmer parameters for warfarin-albumin interaction

Albumin	*T* (°C)	K_{sv}	Intercept	R^2
HSA	25	$(8.46 \pm 0.07)\times10^4$	0.99 ± 0.01	0.9991
	30	$(8.39 \pm 0.07)\times10^4$	1.00 ± 0.01	0.9989
	37	$(8.29 \pm 0.10)\times10^4$	0.99 ± 0.01	0.9983
BSA	18	$(1.29 \pm 0.01)\times10^5$	0.93 ± 0.01	0.9993
	25	$(1.18 \pm 0.01)\times10^5$	0.94 ± 0.01	0.9992
	30	$(1.16 \pm 0.01)\times10^5$	0.95 ± 0.01	0.9988
	37	$(1.08 \pm 0.01)\times10^5$	0.98 ± 0.01	0.9993

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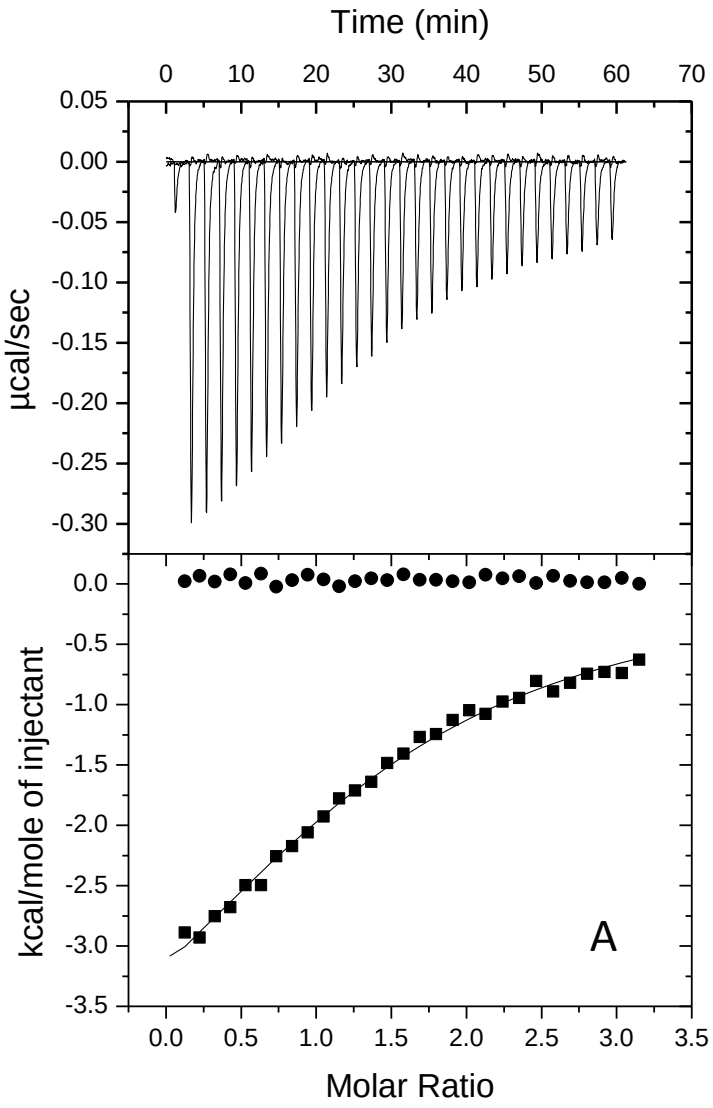
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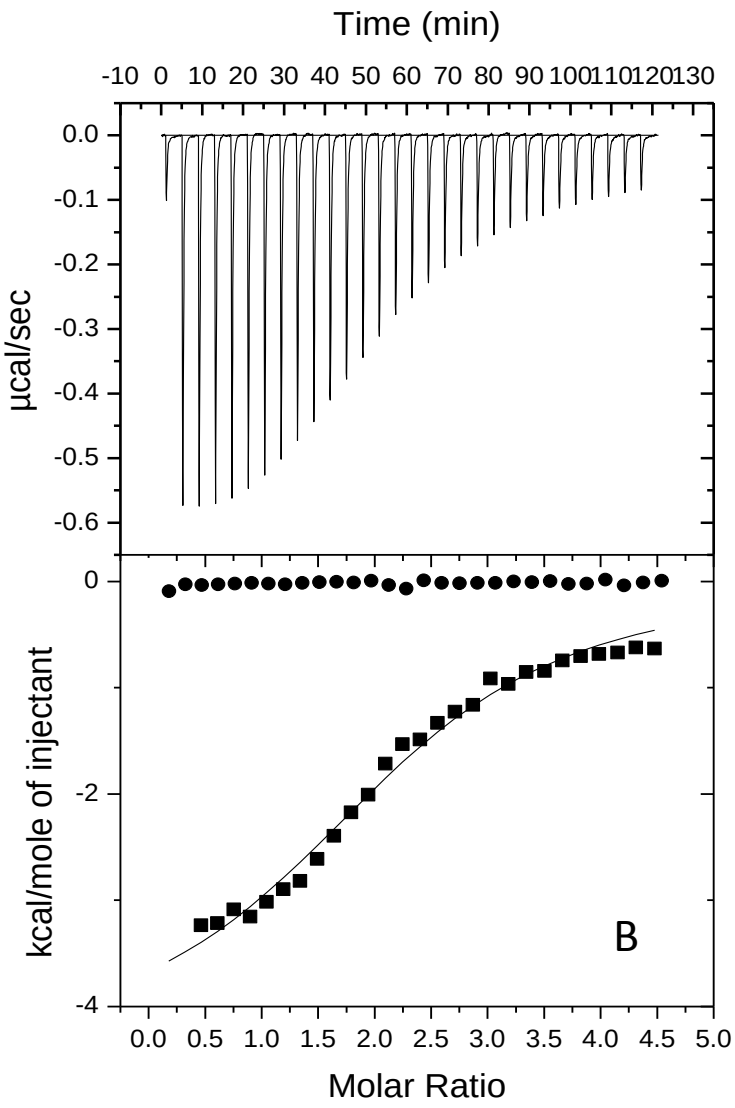
µcal/sec
0.0 -0.5 -1.0 -1.5 -2.0 -2.5 -3.0 -3.5
kcal/mole of injectant
A
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
Molar Ratio


Time (min)
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0.0 -0.1 -0.2 -0.3 -0.4 -0.5 -0.6
µcal/sec
0 -2 -4
kcal/mole of injectant
B
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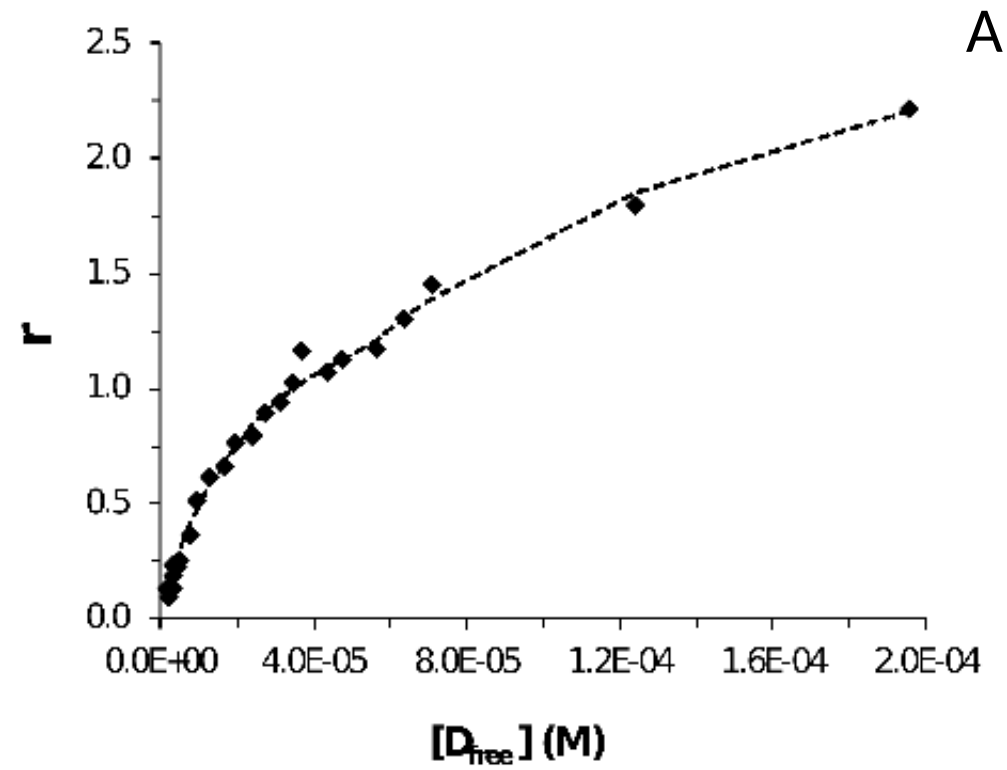
Molar Ratio

Figure 1


A
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2.0
1.5
1.0
0.5
0.0
r
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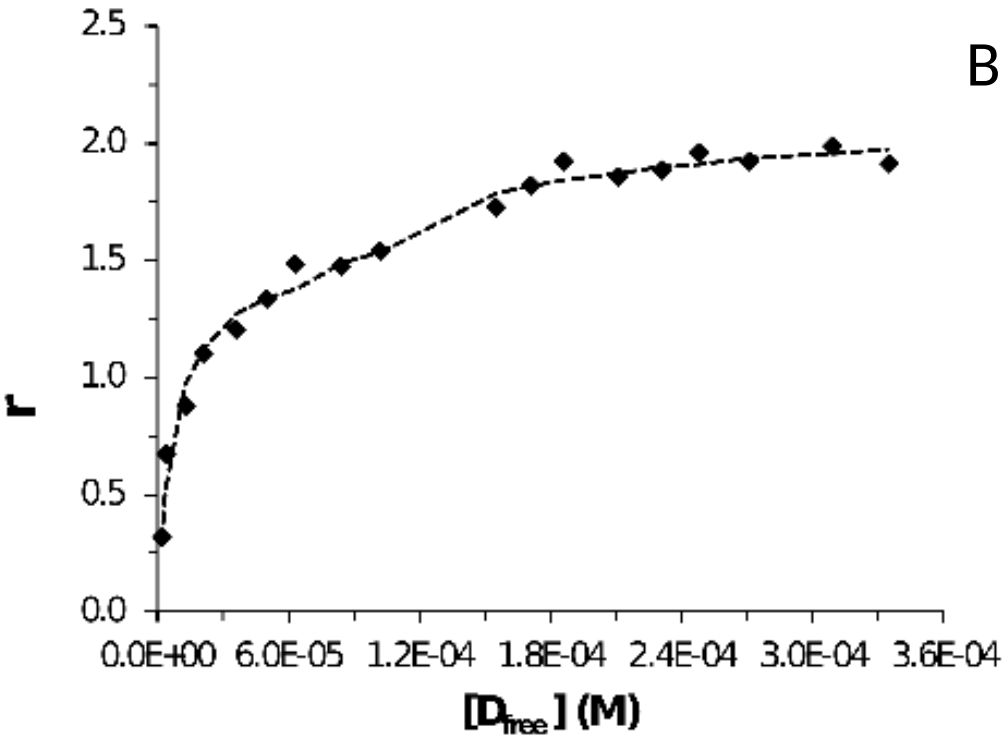
1.6E-04
2.0E-04
[D free] (M)


B
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2.4E-04
3.0E-04
3.6E-04
[D free] (M)

Figure 2

Molecular interactions between warfarin and human (HSA) or bovine (BSA) serum albumin evaluated by Isothermal Titration Calorimetry (ITC), Fluorescence Spectrometry (FS) and Frontal Analysis Capillary Electrophoresis (FA/CE)

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APPENDIX A

According to the following binding process

$P + nD \rightleftarrows D_nP$

the association constant (binding constant) is defined by:

$$K_{ass} = \frac{[D_n P]}{[P_f][D_f]^n} \qquad \text{(Eq. A.1)}$$

where $[D_nP]$, $[P_f]$ and $[D_f]$ are the concentrations of drug-protein complex, free protein and free drug, respectively, being n the number of binding sites.

According to the mass balances

$$[P_T] = [P_f] + [P_b] = [P_f] + [D_nP] \qquad \text{(Eq. A.2)}$$

$$[D_T] = [D_f] + [D_b] = [D_f] + n[D_nP] \qquad \text{(Eq. A.3)}$$

where $[P_b]$ is the concentration of bonded protein and $[D_b]$ is the concentration of bonded drug, and substituting equations (A.1) and (A.2) into equation (A.3), equation (A.4) is obtained:

$$[D_T] = \left(\frac{[P_T] - [P_f]}{K_{ass}[P_f]}\right)^{\frac{1}{n}} + n([P_T] - [P_f]) \qquad \text{(Eq. A.4)}$$

If protein is the only fluorescent component in the considered system, then

$$\frac{F}{F_o} = \frac{[P_f]}{[P_T]} \qquad \text{(Eq. A.5)}$$

where F_o and F are the fluorescence intensities of protein in absence and presence of quencher, respectively.

Substituting equation (A.5) into equation (A.4), the following equation is derived:

$$[D_T] = \left(\frac{F_o - F}{K_{ass} F}\right)^{\frac{1}{n}} + n[P_T]\left(\frac{F_o - F}{F_o}\right) \qquad \text{(Eq. A.6)}$$