CAPIDAN, a Fluorescent Reporter for Detection of Albumin Drug-Binding Site Changes

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Abstract

CAPIDAN, a special fluorescent dye, binds to drug binding sites of human serum albumin. About 90-95 per cent of its fluorescence in serum or plasma originates from albumin-bound dye. This specificity opens a new way to study albumin in unfractionated serum saving intact albumin structure. Due to these properties, CAPIDAN has shown that a variety of diseases is accompanied by some changes of the drug-binding sites. This is a new source of information useful for clinics. That sensitivity to site abnormalities is raised by using time-resolved measurements of fluorescence decay and three new methods of the data analysis: (1) estimation of absolute values of concentration of fluorescent species in heterogeneous biological objects; (2) measurement of dye rotational mobility in heterogeneous albumin binding sites; and (3) method of chloride/nitrate substitution to detect interaction of a drug negative charge with arginine positive charges in the albumin binding sites.

1. Introduction

Measurement of albumin mass concentration in serum is obligatory assay in clinical practice. It is commonly clear that low level of albumin is a negative factor in disease prognosis and is associated with low lifespan. On the other hand, it has been shown that, besides quantity, albumin “quality”, its capacity for binding and transferring a lot of
molecules (different metabolites, fatty acids, hormones, xenobiotics including drugs etc), its ability to clean the body changes due to state of body, and these changes could be very important.

In 1925, it has been found out that a dye, phenol red, binds to albumin much better than to other plasma proteins [1]. Further studies have shown that albumin has much more ability to bind a lot of dyes as well as drugs than other plasma proteins. As a result, human serum albumin (HSA) plays an important role in pharmacokinetics [2-4].

Interest to molecular mechanisms of drug-albumin interaction has raised after the Second World War while it has been found out that penicillin is transported in blood by HSA [3, 5]. In these studies it was shown that the interaction is not covalent, it is reversible, and that drugs and dyes can occupy the same HSA sites competitively. However dyes are quite convenient for that research because their absorption spectra change significantly at binding with HSA [6-8]. Moreover, some dyes including a bromocresol group [9] prefer to bind with HSA in whole plasma (or serum) in spite of the presence of many other proteins. This observation allowed proposing a clinical method of HSA assay which is used today worldwide [10].

However as early as in the 1950-1960 some abnormalities of dye binding have been observed in various diseases. A few researchers started investigation of these deviations. One of the dyes used on the early steps of the research was congo red. Its ability to bind to plasma proteins has been known for a long time (Sorensen 1909: citation from [11]). Csögör has proposed a technique to measure HSA-bound congo red and has shown that its quantity is disease-sensitive [11]. Some other researchers used this technique and confirmed sensitivity of congo red test [12] as well as several other dyes (e.g., [13]). Unfortunately, these methods were rather laborious, therefore systematic clinical studies were not performed.

In 1952, Laurence and Weber have studied interaction of various fluorescent dyes with proteins. An aniline dye, N-phenyl-1-amino-8-sulphonaphthalene (ANS), had very low fluorescence in water, but its fluorescence intensity raised many times in protein, especially albumin solutions [14, 15]. In clinical samples of sera, ANS fluorescence intensity was roughly proportional to HSA concentration but in some samples, especially of jaundice patients, the intensity was decreased [16]. These results had no continuation. Ten years later ANS has been used as a fluorescent probe for studying bovine serum albumin [17-20] and some other proteins [21]. It has been shown that bovine serum albumin has 15 or more binding sites for ANS, but only 5 of them have rather high binding constant of the order of $1 \cdot 10^6 \text{M}^{-1}$ [22].

Following ANS, other fluorescent dyes have been tested to detect HSA changes at pathological processes (e.g., see review [23]). These HSA abnormalities are of significant interest. Therefore a new fluorescent probe named CAPIDAN (or K-35) has been synthesized by B.M.Krasovitsky and colleagues especially for study of HSA pathological changes.

In this chapter, we are focused mainly on physical aspects of CAPIDAN-HSA interaction; a brief review of its clinical testing is proposed also.
2. CAPIDAN: CLINICAL DATA

Human serum albumin represents about 60% of mass of human serum proteins. Its half-life is approximately 20 days [24]. HSA is synthesized in liver [25]. Albumin migrates from plasma to tissues and back, its plasma pool is replaced by the tissue pool during 24 hours [26]. Diseases can upset circulation/distribution of HSA in the body (hepatitis, heart failure, nephrosis) [27, 28].

HSA has a lot of important functions: maintenance of the osmotic pressure [24] and acid/base balance [24; 29], regulation of vascular permeability and hemostasis [30, 31]. HSA can regulate apoptosis [33, 34] and this process depends on the state of HSA thiol groups and presence of some ligands including fatty acids [35]. Albumin is an effective free radical scavenger (both oxygen and nitrogen species) [36].

Another important function of HSA is binding and transport of low-weight molecules (naturally occurring substances: fatty acids [37], bile pigments [38], cholesterol [39], nitrogen oxide [40], cations Zn²⁺, Ca²⁺, Ni²⁺, Cu²⁺, Fe²⁺ [41, 42] and xenobiotics, particularly drugs. Interaction of HSA with free radicals can lead to alteration of albumin binding capacity towards other ligands, including drug substances [43, 44].

Due to binding to HSA, chemical and therefore biological activity of ligand changes. For example, binding of transition valency metals to HSA causes some hundredfold decrease of their activity and capacity to generate radical. Many substances are toxic in free state but after binding to albumin their toxicity significantly lowers [45]. HSA transports these toxicants mainly to the liver where they degrade. Consequently HSA plays quite important role in detoxification system of the body.

Binding of a ligand to albumin molecule occurs in special binding sites. Most endogenous substances have their own special sites (bile and fatty acids, bilirubin, divalent ions). Majority of drugs bind to drug sites I and II. Often competition of different ligands for the same binding place occurs, therefore the actual toxicity of the displaced ligand can increase. Changes of HSA binding capacity in different diseases can enhance toxic effects of drugs and metabolites. Abnormality of albumin distribution between blood stream and other tissues has more pronounced effect on drug pharmacokinetics then simple reduction of albumin concentration [46]. Therefore study of HSA properties particularly of its binding capacity is essential in developing of new drugs.

Albumin Fluorescence Assay and Its Clinical Application

HSA binds different dyes including fluorescent probes.

The fluorescent probe CAPIDAN (N-carboxyphenylimide of dimethylaminonaphthalic acid) was synthesized and kindly gifted by professor B.M.Krasovitsky and colleagues (Institute of Monocrystals, Kharkow, Ukraine). The probe binds to albumin molecule and selectively fluoresces from albumin fraction in the whole serum or plasma [47]. The approximative localization of CAPIDAN in albumin globule is near the Sudlow’s drug-binding site I (see section 5).
A kit to study HSA in its native state (avoiding the fractionating or any treatment of plasma or serum) was developed due to CAPIDAN selective fluorescence. The kit measures two values:

1) effective albumin concentration (EAC) characterizing the quantity as well as the state of binding sites,
2) total albumin concentration (TAC) equal to albumin concentration assayed in clinical laboratories with bromcresol green.

To measure EAC, it is necessary to add the abundance of CAPIDAN to the diluted serum or plasma at pH 7.4. Fluorescence intensity of CAPIDAN under these conditions depends on the state of binding sites which is close to their state in the native plasma without any treatment, on presence of drugs in the “drug” sites or bilirubin or fatty acids etc. In order to determine TAC, we adjusted pH to 4.2 in the same sample and added detergent Brij-35. This procedure provokes “N-F”-transition in HSA globules [48]. Then CAPIDAN fluorescence intensity is approximately proportional to the albumin content and can be converted into the albumin concentration units (g/l). It is considered that in norm EAC comes close to TAC, their ratio EAC/TAC = 1 ± 0.05. EAC/TAC doesn’t depend on concentration and reflects only changes of binding sites and the molecule conformation. Based on these parameters the specific coefficient of HSA binding capacity, “reserve of albumin binding capacity” (RAB), can be calculated according to the following formula: RAB = EAC/TAC x 100%. RAB can be assumed as a measure for a fraction of functionally active HSA, in per cent. However RAB reflects both the binding ability and structural changes of HSA sites.

Numerous studies using this HSA fluorescence assay have shown that decrease of EAC and EAC/TAC accompanies different diseases. The fact has led to the hypothesis that changes of HSA conformation are a special response of the body to pathological processes [49].

Often changes of HSA correlate with the disease form or severity. So the estimation florescent HSA factors can be useful for widening of diagnostics informativity and for the control of treatment efficiency.

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The List of Diseases and Pathological States Which Shown CAPIDAN to Be Quite Informative Is Rather Long

- surgical diseases and critical conditions (peritonitis [50], appendicitis [51], acute pancreatitis, pancreatonecrosis [52-54], burn disease [55], chemical poisoning [56], abscess [57], hernias [58], multitrauma, nurotrauma and critical conditions [59-63]);
- internal diseases (miocardial infarction [64, 65], cardiac failure [66], cerebral ischemia [67], ischemic stroke [68], pneumonia and chronic obstruction lung diseases, hepatitis and hepatic cirrhosis [69], renal failure [70], haemodyalysis, nephropathy, urinemia [71];
- ophhtalmology (glaucoma [72], senele cataract [73] pseudoexfoliation syndrome);
- children diseases and neonatology (multiorgan failure in newborns, kernicterus [74];
- mental disorders (schizophrenia [75-77], depressive syndrome [78-80], and acute poisoning with psychotic drugs [81-83]).

Some results on the usage of the assay or its modifications are described below in more details.

**Myocardial Infarction**

In the begging of the nineties it was shown that immediately after the onset of noncomplicated transmural infraction EAC was normal and during two days decreased for 30%. TAC remained unchanged for two days and lowered for 20% during the next days. In lethal form both factors decreased significantly. In jugulated angor pectoris only EAC lowered but returned to normal values in two days [64].

In [65] the dynamics of EAC and TAC in acute infarction was observed for three weeks. 30% reduction of EAC was found after the onset of the disease and stayed lowered till the end of the monitoring. TAC didn’t vary significantly during the period. Therefore there were little alterations of HSA concentration in acute cardiovascular disorders but CAPIDAN showed conformational abnormalities that occurred during at least three weeks. EAC and EAC/TAC correlated with the severity and form of the disease. As it is shown below (section 10), CAPIDAN fluorescence falls at decrease of HSA-bound fatty acids. Probably these infarction-accompanied changes of HSA properties relate to the abnormalities or failure of fatty acid transportation by HSA.

**Acute Pancreatitis**

Measuring of EAC and TAC can be helpful both to differentiate edematous and necrotising pancreatitis [52] and predict the progression of the disease [54].

![Figure 1. CAPIDAN fluorescence from HSA (effective albumin concentration, EAC) differentiates oedematous and necrotising pancreatitis on the first day of admission.](image-url)
EAC more than 42 g/l at the first day of the admission gave the probability of necrotic form 20%. If EAC was less than 32 g/l the probability of necrotizing pancreatitis grew to 90% (Figure 1). And even a singular decrease of EAC (despite subsequent restoration of the parameter in some cases) during the period of the disease to 14 g/l means the significant raise of the death probability [54].

**Diffuse Peritonitis**

The efficiency of clinical scores for outcome of diffuse peritonitis is not sufficient and the mortality rate in peritonitis remains high. More than 200 patients were examined in a multicentre study [50, 60] and EAC and TAC were compared with results of routine analysis and SAPS and MPI scales. The combination of two parameters – EAC and urea concentration – at the first day of admission was found to be the most useful for outcome prognosis, the efficiency of this combination was very similar to the SAPS data. 95% of benign outcome of diffuse peritonitis followed blood urea level less than 8 mM and EAC more than 14 g/l. High urea (> 8 mM) in combination with low EAC (< 14 g/l) led to benign outcome only in 10% of cases. However the probability of benign outcome was 50% if EAC was between 14 and 22 g/l despite high urea concentration. TAC was less useful for prognosis of diffuse peritonitis. More pronounced alteration of EAC is due to both albumin migration to exudate and tissues and changes of binding sites properties. This can help in an early individual prognosis of diffuse peritonitis.

**Peritoneal Exudation at Peritonitis and Acute Pancreatitis**

In these cases an inflammation in abdominal cavity leads to penetration of plasma proteins including serum albumin from blood into the cavity. The fluorescent method of the total albumin concentration (TAC) assay was used to estimate relationship between albumin concentration in plasma (C_p) and in peritoneal exudate (C_E) [84]. These concentrations have been measured in dynamics of the inflammatory process during several days after admission. In this study it was often detected a significant negative correlation of C_p and the ratio C_E / C_p; i.e., the more the ratio, the lower albumin concentration in plasma. Some examples of this correlation are shown in Figure 2. About half of all patients have this negative correlation at rather high linear coefficient |r| > 0.7; 14 % of patients have r between –0.7 and –0.3.

This strong tendency needs to have a clear statistical support. However it is difficult to do it simply, i.e., by a direct combining all data because of wide variation of personal data. In the patient group of the high negative correlation (51 % of all patients) a linear equation can be approximately used: for i-th patient in j-th measurement

\[ (C_p)_i \approx a_i + b_i(C_E / C_p)_i \]  

(1)

where \( a_i \) and \( b_i \) are personal coefficients. \( a_i \) vary between 16 and 103, \( b_i \) vary between –3 and –65. These individual variations significantly lower the total correlation coefficient. Therefore a procedure has been proposed to unify these data. Step 1: to calculate mean \( a_i \) and mean \( b_i \) for \( m \) patients:
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\[ a = \sum_{i=1}^{i=m} a_i = 47; \quad b = \sum_{i=1}^{i=m} b_i = -23 \]  

(2)

Step 2: each individual \( a_i \) is normalized to the mean \( a \), i.e., shifts of intercepts along \( Y \) axis are averaged; each individual \( b_i \) is normalized to the mean slope of straight lines (see Figure 2), i.e., to \( b \). A normalized function \( f(C_P)_{ij} \) is introduced instead of \( (C_P)_{ij} \):

\[ f(C_P)_{ij} = a_i - (b / b_i) a_i + (b / b_i)(C_P)_{ij} \]

(3)

Figure 2. Three examples of patients with high correlation of plasma HSA concentration \( C_P \) and the ratio Exudate/Plasma, \( C_E / C_P \). Dynamics day-by-day is shown by numbers near experimental points.

Figure 3. Linear correlation of the function \( f(C_P)_{ij} \) and the ratio \( C_E / C_P \) in group of patients with personal \( r_i \) between \(-0.7 \) and \(-1\). The total \( r = -0.94 \pm 0.05 \) at \( p< 0.01 \).
Step 3: a linear correlation coefficient between \( f(C_P)_{ij} \) and the ratio \( C_E/C_P \) is calculated. This transformation does not influence personal coefficients \( r_i \), but allows to do the total tendency more clear. Indeed, each patient in this group has the personal \( r_i \) between \(-0.7\) and \(-1\), however the total \( r \) after that transformation for all this group is very high: it is equal to \( r = -0.94 \pm 0.05 \) at \( p< 0.01 \). So, the tendency of high linear relationship between the ratio \((\text{albumin in exudate})/(\text{albumin in plasma})\) and albumin in plasma is supported by the high correlation coefficient [84]. These relationship can be described well (Figure 3) by the equation:

\[
f(C_P)_{ij} \approx a + b(C_E/C_P)_{ij}
\]  

(4)

Psychiatric Disorders

Shizophrenia

There is an urgent need in tests for diagnostics, differentiation (distinguishing) and prognosis of psychiatric disorders and to estimate the efficiency of psychopharmacotherapy or other therapeutic interventions. In early works on using the CAPIDAN as a probe for clinical purposes it was demonstrated that properties of albumin globule (EAC and EAC/TAC) were significantly altered in these disorders [85, 86]. But the most interesting results were obtained in a study on first-episode drug-naive schizophrenic (FES) patients due to absence of the previous psychopharmacotherapy and thus keeping intactness of HSA binding sites due to loading with antipsychotics and to integrity of metabolism which hadn’t undergone pathological changes yet [87]. Steady-state fluorescence analysis didn’t reveal any changes of HSA site properties [88]. However some modifications of the test medium and analysis of CAPIDAN fluorescence decay showed the significant difference between state of

Figure 4. Effect of change of medium ionic strength on amplitude of the component with decay time 3 ns in donors and first-episode drug-naive schizophrenic patients, \( p < 0.001 \). The data are given as \( M \pm m \). Horizontal axis – ratio of the amplitude at low and physiological ionic strength.
HSA binding sites of the FES patients (ICD-10: paranoid (F20.0) and undifferentiated (F20.3) schizophrenia) and healthy people. CAPIDAN fluorescence decay is complex and has three components with decay time 9 ns, 3 ns and 1 ns (in detail see below section 5). The change of ionic strength of the test medium had different effect on the probe fluorescence decay component with decay time 3 ns in donors or schizophrenics, p < 0.001 [89, 90] (Figure 4).

**Anxious Depression**

As it was shown earlier for schizophrenia (see the previous section), CAPIDAN demonstrated difference between HSA properties of healthy individuals and long-term depression patients [79, 91]. As there is no techniques for early evaluation of the efficiency of therapy of depression and individualization of psychopharmacological treatment can be long enough, HSA fluorescent test has been used for the purpose [80]. 10 depressive patients (F32.1 (depressive episode as an independent disease) according to ICD-10) were under observation for three – four weeks of psychopharmacological treatment. There were no any therapeutic intervention before the begging of the study. CAPIDAN fluorescence decay analysis (in details see below in section 5) revealed that mean fluorescence intensity of the probe in patient HSA before the beginning of the treatment is lower than in healthy donors, p <0.01. CAPIDAN fluorescence decay is complex and has three components with decay time 9 ns, 3 ns and 1 ns (see below, section 5). Figure 5 shows amplitudes of the 9 ns decay component of CAPIDAN in patients’ HSA before and within three weeks of psychopharmacotherapy. Almost all patients showed an increase of amplitude “9 ns” after the treatment (p<0.01 according to Wilcoxon's criterion for coupled variants). Mean raise of amplitude “9 ns” after the treatment was 15%. So HSA fluorescent data is rather sensitive to pathological process in anxious depression and can reflect HSA changes due to psychopharmacological treatment.

![Figure 5. Change of amplitude “9 ns” of CAPIDAN fluorescence decay in patients with anxious depression after three weeks therapy. Amplitude is expressed in arbitrary units.](image-url)
Acute Poisoning with Psychotropics and Its Complications

HSA fluorescence test (a steady-state version, see below, section 4) was compared to usual laboratory tests for endotoxemia (a hematology and biochemistry panel, middle molecules) in patients (n=513) with acute overdose with psychotropics [81]. On the first day of admission HSA fluorescence test was the best to predict lethal cases in the patients with medium and severe poisoning. Pneumonias often complicates the disease and results in 60% mortality. Clinical evidence of the pneumonia is opaquing, particularly at the beginning of the disease, that complicates pneumonia prognosis. CAPIDAN fluorescence intensity in HSA of the patients with following pneumonia is often lower than in patients without pneumonia. This difference helps to select a group of subjects just having or at risk of following [82].

Thus HSA fluorescence test with CAPIDAN and its modifications can be of prognostic and diagnostic value in many pathologies.

3. CAPIDAN MOLECULE

CAPIDAN molecule is shown in Figure 6. It contains a carboxylic group which losses H+ in water solutions; its pKₐ is near 4.2, therefore CAPIDAN molecule is negatively charged at pH 7. As it is known, negatively charged dyes bind to HSA better than uncharged and positively charged ones [7, 8]. This CAPIDAN carboxyl should be localized on the boundary “binding site/water phase” due to hydration the negative charge by water.

Figure 6. CAPIDAN molecule. Ionic form in water solutions at pH 7. Numeration of atoms is shown.

3.1. Quantum Chemical Data

Bond lengths and atom charges have been calculated recently in solvents of different polarity [92]. As can be seen, CAPIDAN molecule is not nonpolar and hydrophobic. Intermolecular charge shift (Table 1) rises creates dipole moments. Two important moments are originated from charge transfer in two groups O=C=N; therefore one can expect that this fragment of the molecule has to be solvated by polar environment. The charge shift along the long axis of the molecule is responsible for a general dipole moment of about 10 D. This moment is very important for absorption and fluorescent spectra shifts in polar media as well as in HSA (details see below). These three moments are shown in Figure 7.
All these charge shifts raise in polar environment (Table 1).

**Table 1. CAPIDAN atom charges as natural bond orbital (NBO) in solvents of different polarity: computer simulation. ε is the dielectric constant. For details see [92]**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Heptane</th>
<th>Acetone</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent polarity ε</td>
<td>1.9</td>
<td>22</td>
<td>80</td>
</tr>
<tr>
<td>Atom</td>
<td>Charge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C_6</td>
<td>0.21</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>N_20</td>
<td>0.08</td>
<td>0.14</td>
<td>0.15</td>
</tr>
<tr>
<td>C_15</td>
<td>0.41</td>
<td>0.49</td>
<td>0.52</td>
</tr>
<tr>
<td>O_21</td>
<td>−0.70</td>
<td>−0.73</td>
<td>−0.74</td>
</tr>
<tr>
<td>C_16</td>
<td>0.63</td>
<td>0.71</td>
<td>0.74</td>
</tr>
<tr>
<td>O_22</td>
<td>−0.70</td>
<td>−0.74</td>
<td>−0.74</td>
</tr>
<tr>
<td>C_27</td>
<td>−0.59</td>
<td>−0.56</td>
<td>−0.56</td>
</tr>
<tr>
<td>N_35</td>
<td>−0.15</td>
<td>−0.17</td>
<td>−0.16</td>
</tr>
<tr>
<td>C_36</td>
<td>−0.38</td>
<td>−0.39</td>
<td>−0.39</td>
</tr>
<tr>
<td>C_40</td>
<td>−0.37</td>
<td>−0.38</td>
<td>−0.38</td>
</tr>
<tr>
<td>C_31</td>
<td>−0.58</td>
<td>−0.63</td>
<td>−0.65</td>
</tr>
<tr>
<td>C_33</td>
<td>−0.28</td>
<td>−0.27</td>
<td>−0.27</td>
</tr>
<tr>
<td>N(CH_3)</td>
<td>−0.14</td>
<td>−0.13</td>
<td>−0.12</td>
</tr>
</tbody>
</table>

Figure 7. Dipole moments (arrows) in CAPIDAN molecule.

So, the aromatic part of the molecule can not be assumed as nonpolar and hydrophobic. Meanwhile perhaps this fragment significantly contributes to the binding energy in the case of CAPIDAN-HSA complexes. Estimation of the energy changes due to transfer of this aromatic fragment from water to nonpolar heptane has shown that this transfer requires 10 kcal/mol (i.e., is almost impossible) while the transfer from water to polar acetone gives a profit of 5 kcal/mol [92]. This profit is due to great energy of cavity formation for the fragment in water as compared with acetone. So, the aromatic fragment has poor solubility both in nonpolar media and in water but is well soluble in media of moderate polarity.
3.2. Proton Magnetic Resonance Data

CAPIDAN proton magnetic resonance spectra in water show narrow lines in 6–9 ppm area attributed to hydrogen atoms of the aromatic system, and a narrow line of 6 hydrogen atoms of the dimethylamino group at 3.0 ppm [92]. This amino group can rotate about the bond N35–C27 but this rotation has a steric barrier: it needs ≈10 kcal/mol for the methyl group overcomes the hydrogen atom H–C33 [92].

4. Steady State Fluorescence

In the begging CAPIDAN-based fluorescent test was used in its steady-state version. To understand a general picture of the dye molecule environment in HSA binding sites, it has been performed a study of absorption and fluorescence spectra in a variety of solvents (Table 2). The first factor important for the spectral maxima is solvent polarity. Its magnitude can be estimated by the Lippert’s function $f(\varepsilon, n)$ [93, 94]:

$$f(\varepsilon, n) = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}$$

Here $n$ is the refractive index and $\varepsilon$ is the dielectric constant. This function describes orientation of solvent dipolar molecules near solute dipoles. CAPIDAN molecule has dipoles (Figure 7), and therefore there is a dipole-dipole interaction the dye-solvent which leads to formation of a solvent shell of oriented dipolar molecules around the dye.

In nonpolar solvents, one needs about 70 kcal/mol to excite the CAPIDAN molecule (Table 2). The more solvent polarity, the more $f(\varepsilon, n)$, the less the excitation energy and the spectral maxima of absorption, fluorescence excitation and emission (Table 2 and Figure 8). So, solvation of the dye by polar molecules leads to decrease of the dye excitation energy.

Table 2. Absorption, excitation and emission spectral maxima of CAPIDAN in solvents of different polarity and in HSA water solutions at 25°C. Mean error of the maximum position is ± 1.5 nm

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Polarity (f(\varepsilon, n))</th>
<th>Absorption maximum nm</th>
<th>Absorption energy kcal/mol</th>
<th>Excitation maximum nm</th>
<th>Emission maximum nm</th>
<th>Stokes' shift nm</th>
<th>Half-width of emission nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>0.02</td>
<td>407</td>
<td>70.5</td>
<td>402</td>
<td>497</td>
<td>90</td>
<td>69</td>
</tr>
<tr>
<td>o-xylene</td>
<td>0.04</td>
<td>412</td>
<td>69.7</td>
<td>404</td>
<td>497</td>
<td>85</td>
<td>71</td>
</tr>
<tr>
<td>pentachlorhexane</td>
<td>0.14</td>
<td>397</td>
<td>72.3</td>
<td>418</td>
<td>502</td>
<td>105</td>
<td>70</td>
</tr>
<tr>
<td>chloroform</td>
<td>0.15</td>
<td>420</td>
<td>68.3</td>
<td>420</td>
<td>507</td>
<td>87</td>
<td>74</td>
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<tr>
<td>isobutanol</td>
<td>0.26</td>
<td>423</td>
<td>67.8</td>
<td>425</td>
<td>535</td>
<td>112</td>
<td>82</td>
</tr>
<tr>
<td>N,N-dimethylformamide</td>
<td>0.27</td>
<td>425</td>
<td>67.5</td>
<td>425</td>
<td>533</td>
<td>108</td>
<td>81</td>
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<tr>
<td>Acetone</td>
<td>0.28</td>
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<td>69.2</td>
<td>425</td>
<td>522</td>
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<tr>
<td>Ethanol</td>
<td>0.29</td>
<td>426</td>
<td>67.4</td>
<td>428</td>
<td>538</td>
<td>112</td>
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<tr>
<td>Glycerol</td>
<td>0.26</td>
<td>448</td>
<td>64.1</td>
<td>449</td>
<td>526</td>
<td>77</td>
<td>87</td>
</tr>
<tr>
<td>water</td>
<td>0.32</td>
<td>448</td>
<td>64.1</td>
<td>448</td>
<td>537</td>
<td>89</td>
<td>97</td>
</tr>
<tr>
<td>human albumin</td>
<td>–</td>
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<td>64.1</td>
<td>448</td>
<td>521</td>
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<td>72</td>
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</tbody>
</table>
However this excitation energy is extremely low in the case of the HSA-bound dye: the decrease is 6 kcal/mol as compared with nonpolar media. Exactly the same energy takes place in water and glycerol. This comparison leads to suggestion that a general explanation for these three cases is the existence of hydrogen bonds solvent-solute as $\text{–O–H...O=dye}$. So, the dye carbonyl group or both carbonyls form hydrogen bonds in HSA binding site.

If excitation changes the dye dipole moment, it has to induce a reorganization of environmental dipoles. The reorganization needs energy, its source is the energy of the excited state. As a result, the emission maximum shifts to longer wavelength. Therefore the Stokes’ shift can be more in polar media. But HSA-bound CAPIDAN has the least Stokes’ shift (Table 2). Perhaps movement of polar molecules near the dye in the binding site is strongly hindered, they can not rotate in excited state life time. This hypothesis is supported by the data on rotation of the dye in HSA sites (see below, section 7.2).
Structure of the polar solvation shell in polar solvents is rather variable; therefore the Stokes’ shift is variable too. This phenomenon leads to widening the total emission spectrum. Indeed, the half width is about 70 nm in the case of nonpolar solvents and raises to 87 nm in polar ones (Table 2). However this dye in HSA binding sites has the same excitation maximum as in polar proton-donor solvent but an extremely narrow emission peak, 72 nm, like to nonpolar media. It can be suggested that environment of the dye in HSA is not so variable as in polar solvents, i.e., this environment in HSA has a certain structure. But nevertheless some variations take place: emission spectrum slightly moves to long wavelengths if excitation wavelength raises (Figure 9).

Degree of fluorescence polarization of HSA-bound CAPIDAN is independent of emission wavelength (Figure 10). This fact shows that the emission can be attributed to a single electronic transition at any wavelength. High anisotropy of polarized fluorescence in the excitation moment, \( r(0) \), close to its theoretical maximum, 0.4, means that orientation of absorption and emission dipoles is almost similar, probably along the molecule long axis.

![Figure 10. Anisotropy of polarized fluorescence, \( r(0) \) (in the first moment after excitation), of HSA-bound CAPIDAN. Excitation 400 nm.](image)

5. **TIME-RESOLVED FLUORESCENCE**

Steady-state fluorescence gives an interesting information on CAPIDAN environment in HSA binding sites, and many researchers use some versions of that techniques of fluorescence measurements to study of biological objects [95]. However these objects are as a rule rather complex, while steady-state fluorescence is a source of rather limited information. Moreover, in some cases the steady-state data give an incorrect picture. e.g., addition of phenylbuthazone, a marker of HSA drug-binding site I, to CAPIDAN-HSA complexes decreases CAPIDAN steady-state fluorescence intensity and leads to an impression that these complexes dissociate; however more detailed study shows that the complex does not dissociate but the probe environment is changed in the HSA molecule (see below section 5.4).
Much more information can be obtained in the study of fluorescence decay by the time-resolved techniques.

5.1. Simplest Cases of Fluorescence Decay

In simplest cases, there is a rather diluted solution of fluorescent molecules where environment of each molecule can be assumed as the same. $C$ is their concentration. After illumination by a short light flash a fraction of these molecules absorbs light quanta and becomes excited. Their initial concentration is $C_0$ but it gradually decreases as a time function $C^*(t)$ due to deactivation processes. The small decrease, $-dC^*(t)$, of this concentration in a small time interval $dt$ is assumed to be proportional to the concentration:

$$\frac{-dC^*(t)}{dt} = k_D C^*(t);$$

after integration: $C^*(t) = C_0^* \exp(-k_D t) = C_0^* \exp(-t/\tau_D)$ (6)

Here $k_D$ is the rate constant of the deactivation and $\tau_D = 1/k_D$ is its decay time. In this simple case, three major processes can be assumed as causes of this deactivation. The first one is fluorescence emission (i.e., radiative transitions); its rate constant is $k_F$. The second one is intramolecular processes without fluorescence emission (i.e., nonradiative transitions); this rate constant is $k_N$. Finally, processes of interaction with some outer molecules could lead to deactivation, and, as a consequence, to partial fluorescence quenching; their rate constant is $k_Q$. As a result, $k_D$ is the sum of these three constants:

$$k_D = k_F + k_N + k_Q$$ (7)

Fraction of excited molecules deactivated in time $dt$ by fluorescence emission is $dC_F^*(t) = (k_F / k_D) C^*(t) dt$. Fluorescence intensity $F(t)$ is proportional to the derivative $dC_F^*(t)/dt$:

$$F(t) = b_c \frac{-dC_F^*(t)}{dt} = b_c \frac{k_F}{k_D} C_0^* \exp(-k_D t) = b_c k_F C_0^* \exp(-k_D t)$$ (8)

where $b_c$ is a coefficient dependent on parameters of the instrument used. The more two nonradiative rate constants, $k_N$ and $k_Q$, the less fluorescence intensity. Preexponential factor can be named amplitude, $A$, of this exponential:

$$A \equiv b_c k_F C_0^*; \quad F(t) = A \exp(-k_D t) = A \exp(-t/\tau_D)$$ (9)

It is interesting that both values, exponential and amplitude, depend on the radiative rate constant $k_F$.

This simple case can be illustrated by fluorescence decay of a fluorescent probe, 3-methoxybenzanthrone (MBA) [95, 96]. It has monoexponential fluorescence decay (straight
line in Figure 11, semi logarithmic axes) with $\tau_D = 12.5$ ns and $k_F = 4.8 \cdot 10^7$ s$^{-1}$ in N,N-dimethylformamide.

![Figure 11. Fluorescence decay of MBA and HSA-bound CAPIDAN. MBA is solved in N,N-dimethylformamide. Three components of the CAPIDAN decay are shown also. Logarithmic vertical scale.](image)

However fluorescence decay of HSA-bound CAPIDAN is not exponential (nonlinear curve in Figure 11). Obviously, either its different molecules have different environment in HSA sites or their interaction with environment in HSA molecule has a complex character.

### 5.2. Complicated Fluorescence Decay

Fluorescence decay of the HSA-bound CAPIDAN is described by a nonlinear curve in Figure 11, i.e., this deactivation process is not simple. This curve depends on probe and HSA concentrations, pH, ionic contents of solvents etc. Therefore the curve needs formalism for description of its shape. A convenient way to its description is a sum of exponentials:

$$F(t) = \sum_{i=1}^n A_i \exp(-t / \tau_i)$$  \hspace{1cm} (10)

In the case of HSA-CAPIDAN system, the sum of one or two exponentials can not describe satisfactory this decay curve while three exponentials give a good, satisfactory description [23, 92].
Table 3. Decay times $\tau_1$, $\tau_2$, $\tau_3$ of albumin-bound CAPIDAN at variation of amplitude ratio $A_3/A_1$ due to change of ionic strength ($I$), phenylbutazone (Ph) and palmitate (Pal) addition. pH 7.4. Mean error of $\tau_i$ is near $\pm 0.1$ ns. Data on fatty acid -free HSA and on sera of donors

<table>
<thead>
<tr>
<th>Sample</th>
<th>$I$ mol/Litre</th>
<th>Ph /HSA, mol/mol</th>
<th>Pal /HSA mol/mol</th>
<th>$A_3 / A_1$</th>
<th>$\tau_1$ ns</th>
<th>$\tau_2$ ns</th>
<th>$\tau_3$ ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td>0.85</td>
<td>8.9</td>
<td>3.4</td>
<td>1.0</td>
</tr>
<tr>
<td>HSA</td>
<td>0.19</td>
<td>0</td>
<td>0</td>
<td>1.80</td>
<td>8.8</td>
<td>3.1</td>
<td>0.8</td>
</tr>
<tr>
<td>HSA</td>
<td>0.19</td>
<td>2/1</td>
<td>0</td>
<td>6.7</td>
<td>8.5</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td>HSA</td>
<td>0.19</td>
<td>0</td>
<td>3/1</td>
<td>7.7</td>
<td>8.7</td>
<td>2.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Serum of donor 1</td>
<td>0.19</td>
<td>0</td>
<td>–</td>
<td>2.6</td>
<td>8.5</td>
<td>3.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Serum of donor 2</td>
<td>0.19</td>
<td>0</td>
<td>–</td>
<td>5.0</td>
<td>8.7</td>
<td>3.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

However are these three exponentials an expression of some real physical processes, or it is a product of a formal mathematical procedure? If this is a mathematical artifact, then in different conditions (concentrations, pH etc.) different sets of parameters $\tau_i$, $A_i$ will take place; more over, $\tau_i$ would be dependent on contribution of each component, i.e., on $A_i$. Data in Table 3 shows that decay times $\tau_i$ are almost independent of $A_i$ at very wide range of these amplitudes. So, parameters $\tau_i$, $A_i$ in the sum of Equation 10 are not a product of mathematical manipulations; they reflect actual existence of three different fluorescent species. $\tau_1$ value is close to 9 ns, $\tau_2$ is near 3 ns and $\tau_3$ is about 1 ns. Two first species are responsible for about 90-95% (40-50% each) of total fluorescence of HSA-bound CAPIDAN. This conclusion is supported by the data described below (sections 5.4 and others). Decay times are rather similar in isolated HSA and in intact donor sera (Table 3).

5.3. Estimation of Absolute Concentrations of Fluorescent Species

Equation 10 describes fluorescence decay of those molecules whose decay can be measured. Meanwhile the existence of molecules with strongly quenched fluorescence which is not measured due to their very low decay time can not be excluded. This is a problem. The second problem is the estimation of absolute concentrations of each fluorescent species. e.g., CAPIDAN fluorescence can be represented as the sum of several fluorescent species (Equation 10). However there should be a way to estimate concentrations of these species. It is clear that decay amplitudes $A_i$ reflect these concentrations: the more the concentration, the more the amplitude (Equation 9). But amplitudes depend also on the rate constant $k_F$ and instrument parameters.

A way to solve this problem has been proposed recently [95, 96]. In brief, a fluorescent sample studied is compared with a standard sample. There is a sample studied where concentration of the fluorescent species is $C$, its molar extinction at excitation wavelength is $\varepsilon$, fluorescence intensity in steady-state spectrum at wavelength $\lambda$ is $\Phi(\lambda)$ and $S\Phi$ is integral of its steady-state fluorescence spectrum. Fluorescence decay is measured at $\lambda$. Also there is a standard solution (subscript “s”) of the same chromophor with known concentration $C_S$ and
known parameters $\varepsilon_S$, $\Phi_S(\lambda)$, $S\Phi_S$. Their fluorescent rate constant are $k_F$ and $(k_F)_S$, respectively. Instrument sensitivity is $b_c(\lambda)$. Approximately $\varepsilon \approx \varepsilon_S$. Then

$$A(\lambda) = \frac{b_c(\lambda)k_F C}{b_c(\lambda)(k_F)_S C_S} \frac{\Phi_S(\lambda)/S\Phi}{(k_F)_S C_S \Phi_S(\lambda)/S\Phi} \approx \frac{k_F C}{(k_F)_S} \frac{\Phi_S(\lambda)/S\Phi}{\Phi_S(\lambda)/S\Phi}$$

(11)

Concentration $C$ which needs to be measured is equal to

$$C \approx C_S \frac{A(\lambda) (k_F)_S \Phi_S(\lambda)/S\Phi}{A_S(\lambda) k_F \Phi(\lambda)/S\Phi}$$

(12)

If chromophor is the same in both samples, then $k_F$ and $(k_F)_S$ could be assumed as the same, $k_F/(k_F)_S \approx 1$, and all parameters in the right part of this equation are known, then the concentration $C$ can be calculated. However if chromophors are different, then $k_F$ and $(k_F)_S$ are different too, and their ratio is indefinite. This case can be solved [95] using Einstein-Strickler-Berg expression for $k_F$ [97, 98]:

$$k_F \approx \text{const} \frac{1}{(\lambda_M)^{\beta}}$$

(13)

where $\lambda_M$ is maximum of fluorescence spectrum and $\beta \approx 3$; $\text{const}$ is a combination of physical constants. This is a more general case, and Equation 12 can be modified for practical use:

$$C \approx C_S \frac{A(\lambda) \lambda_M^{3} \Phi_S(\lambda)/S\Phi}{A_S(\lambda) \lambda_M^{3} \Phi(\lambda)/S\Phi}$$

(14)

where $\lambda_M$ and $(\lambda_M)_S$ are maximums of fluorescence spectra of the fluorescence species studied and a standard sample, respectively. This formalism has been tested using 3-methoxybenzanthron (MBA) in organic solvents and lipid membranes and has shown satisfactory results [95]. A test has been made for the CAPIDAN case; its results are presented in Table 4. A model solution of CAPIDAN in glycerol shows that CAPIDAN fluorescence decay in glycerol is nonexponential and can be described as a sum of several exponentials (as in Equation 10). Averaged decay time

$$<\tau> = \frac{A_1\tau_1 + A_2\tau_2 + A_3\tau_3}{A_1 + A_2 + A_3}$$

(15)

decreases in glycerol heating. In spite of dramatic changes of the decay due to heating, the sum of amplitudes, $A_1 + A_2 + A_3$, is not changed in rather wide temperature interval. This fact could be a sign that in this temperature interval all dye molecules are fluorescent (they have decay time more than 0.2 ns). To check this hypothesis, the approach described above was used, with MBA in ethanol as a standard sample. As can see in the Table 4, the data are consistent with that hypothesis: fraction of fluorescent molecules is close to 1.0 (at T>40 °C
this fluorescent fraction decreases because of appearing molecules with short decay time <0.2 ns). So, this approach can be used for estimation absolute concentrations of CAPIDAN fluorescent species.

Table 4. Fluorescence decay of CAPIDAN in glycerol at temperature variations. $\langle \tau \rangle$ is a mean decay time (see Equation 15), $f(T)$ is a fraction of fluorescent molecules having partial decay time >0.2 ns. Mean error of $\langle \tau \rangle$ is ± 0.1 ns, $f(T)$ is ± 0.13

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>$\langle \tau \rangle$, ns</th>
<th>$f(T)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td>21</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>30</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>40</td>
<td>0.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

5.4. Heterogeneity of Albumin Binding Sites

As it is known, the HSA molecule is able to adsorb several aromatic dye molecules [99] but two special binding sites have the higher affinity to drugs and dyes [100] (Figure 12).

![Figure 12](image.png)

Figure 12. Localization of domains, subdomains (Roman numerals), two major drug-binding sites (arrows) and long-chain nonesterified fatty acids (rhombs with numbers) in HSA crystals. On the base of data of [101-103].

The site I is accessible for molecules of more size as compared with site II. Where does CAPIDAN localize? Why does three fluorescent species originate? Some data presented below lead to conclusion that the main place of its localization is the drug site I however interaction of the dye with this site I has different forms.

Three Fluorescent Species: Different Preexisting Environments

Steady-state fluorescence has shown that different molecules of HSA-bound CAPIDAN have different environment polarity (Figure 9). Indeed, these data are supported by time-resolved fluorescence spectra. Fluorescence decay demonstrates existence of three fluorescent species (see above). They have different decay time $\tau_i$. When does this difference rise: during
excited state or before it? Excitation spectra show that the species 1 has maximum of the spectrum near 445 nm, species 2 – near 448 nm and species 3 – near 553 nm. So, molecules responsible for these species interact with their environment differently before excitation; species 3 has the most polar solvation shell (or more strong H-bonds) than other species; the less $\tau_i$, the more this polarity. This fact leads to suggestion that variation of the decay time $\tau_i$ may be a consequence of a different structure of polar interactions of the dye with the occupied site. May be, this polarity originates from water molecules which localize into the site I and quench dye fluorescence. This hypothesis is supported by some data described below.

![Figure 13](image)

**Figure 13.** Fractions of the three species of HSA-bound CAPIDAN as a function of pH. Each fraction is normalized on its value at pH 7.4.

**Three Fluorescent Species: Similarity of Their General Localization**

As it is known, pH change induces conformational transitions in HSA molecule, so-named N–F at pH near 3-5 and N–B at pH 8–10 [99]. Fluorescence of the dye also is sensitive to these transitions (Figure 13). It is interesting that these albumin conformational transitions influence all three species similarly. Perhaps, the dye molecules responsible for these species localize in the same sites of HSA globule.

**CAPIDAN Competition with a Marker of the Site I.**

An anti-inflammatory drug, phenylbutazone, is known as a marker of the drug-binding site I [99, 103]. Addition of phenylbutazone to HSA leads to decrease of CAPIDAN steady-state fluorescence [23]. This fact can be a sign of competition of these two ligands in the site I.

The simplest scheme of this competition might be so: phenylbutazone occupies the site, therefore the dye can not bind to the site. However time-resolved data show more interesting events. The drug influences all three fluorescence species. Three decay times are not changed (Table 3). Amplitudes of the first two species, i.e., $A_1$ and $A_2$, decrease, therefore quantity of dye molecules of this kind indeed decreases. At the same time $A_3$ increases. As a result, the sum $A_1+A_2+A_3$, which represents a total number of HSA-bound dye molecules of these species, stays approximately the same. So, phenylbutazone changes the dye environment in
CAPIDAN, a Fluorescent Reporter for Detection of Albumin Drug-Binding …

the site I but does not prevent its binding to albumin. Perhaps, species 1 and 2 are converted into the species 3. Therefore decay times 9 and 3 ns are converted into 1 ns leading to fluorescence intensity decrease (Table 3).

More details of phenylbuthazone-CAPIDAN relations are described below, see Table 5.

**Coulomb Interaction in the Binding Sites**

All decay amplitudes of bound CAPIDAN are sensitive to ionic strength, $I$, of the solution (Table 3): the more $I$, the less amplitudes. It can mean that the negatively charged carboxyl group of the dye localizes on the boundary “site/water phase” close to some positive charges. The species have different sensitivity to the ionic strength: $A_1 > A_2 > A_3$. Amplitudes present the bound dye molecules, the binding process needs much more time than fluorescence decay time $\tau$. So, there is a Coulomb interaction dye-albumin, but three species have different strength of the interaction. This interaction and this difference are formed long before dye excitation. In other words, the difference of environment of these three fluorescent species preexists, this is not a result of excitation.

At pH raise from 7 to 9 aminogroups of lysine residues loss their positive charge. At the same time binding of this negatively charged dye significantly increases (raise of all three amplitudes on Figure 13). So, there is no evident Coulomb interaction of the dye with lysines. Perhaps, the Coulomb interaction can be attributed to positively charged arginine residues. This hypothesis is supported by some reasons described below (section 8).

**Binding Parameters of the Complex CAPIDAN-HSA**

The simplest attempt to propose a formalism for the dye reversible interaction with HSA can be based on a suggestion that there exist three state of the complex CAPIDAN-site I. As a result, three fluorescent species appear. HSA concentration is $C_A$. The mean number of each type of sites able to form these complexes is $n_1$, $n_2$ and $n_3$ per one HSA molecule, respectively. Concentration of dye molecules formed each type of these complexes is $r_1$, $r_2$ and $r_3$, respectively. Concentration of free dye is $c$. Then the simplest system of three equations for this equilibrium is

$$K_i = \frac{r_i}{c \cdot (n_i C_A - r_i)}, \quad i = 1, 2, 3. \quad (16)$$

where $K_i$ are the binding constants for these binding sites. Values of $r_i$ are measured by the method of amplitude standard described above. This method permits to estimate a constant $b$ in equations

$$r_1 = bA_1; \quad r_2 = bA_2; \quad r_3 = bA_3 \quad (17)$$

where $b$ has the same value for all three species. Amplitudes $A_1$, $A_2$ and $A_3$ are taken from decay kinetics (Equation 10).

Data on binding parameters in CAPIDAN-HSA complexes are presented in Table 5. The first species has the most $K$. The site I marker, phenylbuthazone, suppresses dye binding with
the site decreasing both $K_1$ and $n_1$. This marker lowers also $n_2$ and $n_3$ but strongly increases $K_3$. As a result, the rough estimate of HSA affinity to the dye

$$\text{Affinity} \approx K_1 n_1 + K_2 n_2 + K_3 n_3$$  \hspace{1cm} (18)$$

is almost the same without as well as with the marker while it strongly lowers total fluorescence intensity: a rough calculated estimate for the total intensity, $F_C$, can be presented as

$$\text{Fluorescence } F_C \approx b_F (K_1 n_1 \tau_1 + K_2 n_2 \tau_2 + K_3 n_3 \tau_3)$$  \hspace{1cm} (19)$$

Presence of phenylbuthazone lowers this calculated $F_C$ by 43%. This is close to the experimental value 45%. It is interesting that three partial affinities $K_i n_i$ without this marker have almost the same values (Table 5), i.e., all three species have almost the same concentrations at low ratio drug/HSA. Phenylbutazone shifts this equilibrium to the third species.

So, this simple model of HSA-CAPIDAN equilibrium allows to describe the general facts on concentrations of three bound species. More complex model takes into account a dependency of $K_i$ on degree of site occupation by the dye [104]: $K_2$ and $K_3$ increase at occupation raises, i.e., there is a sign of positive cooperativity of the binding (however other hypotheses can be proposed too).

Table 5. Binding parameters of CAPIDAN-HSA complexes. Fatty acid-free HSA concentration is 25 μM, ionic strength 0.16 M, pH 7.4. Ratio phenylbuthazone (Ph)/HSA is 0 or 1. Mean relative error of $K$ and $n$ values is about ±10 %, for $Kn$ values is ±13 %

<table>
<thead>
<tr>
<th>Ph/HSA mol/mol</th>
<th>Parameter</th>
<th>Species</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K$</td>
<td>1 (9 ns)</td>
<td>2 (3 ns)</td>
<td>3 (1 ns)</td>
</tr>
<tr>
<td>0</td>
<td>45000</td>
<td>10000</td>
<td>6000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$n$</td>
<td>0.12</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>$Kn$</td>
<td>5400</td>
<td>6800</td>
<td>6600</td>
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</tr>
<tr>
<td></td>
<td>$n$</td>
<td>0.08</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>$Kn$</td>
<td>2100</td>
<td>5000</td>
<td>9600</td>
</tr>
</tbody>
</table>

6. CAPIDAN SOLVATION INSIDE THE BINDING SITE

About 63 years ago, Laurence and Weber have proposed the first fluorescent probe, N-phenyl-1-amino-8-sulphonaphthalene (ANS), for studying HSA. ANS has very low fluorescence in water, but its fluorescence intensity raises many times in HSA solutions [14, 15]. CAPIDAN has the same property which is convenient for HSA study: water quenches its fluorescence, so in water the dye has a low decay time $\tau_W < 0.2$ ns. Binding to HSA hides the dye from water, and decay time raises. At the same time a fraction of the HSA-bound dye is partially quenched: its decay times, $\tau_2 \approx 3$ ns and $\tau_3 \approx 1$ ns, are significantly lower than the
high value of $\tau_1 \approx 9$ ns. What is the quencher? What is the quenching mechanism in water and in HSA?

Both steady-state and time-resolved techniques show (see above) that all three species of bound dye molecules have very polar environment and/or H-bonds with environment, as in water. It is probable, some water molecules penetrate the binding site and solvate the dye molecule. However a simple solvation by dipole-dipole interaction is not able to shift absorption and excitation spectra to the red side so strongly (Table 2). Therefore this shift could be a result of H-bonds between water molecules and O=C groups of the aromatic chromophor, i.e., HO–H...O=C–dye.

Can the polar medium and/or H-bonds be a major cause of the partial fluorescence quenching of the bound dye? High environment polarity shifts electron levels in chromophors having dipole moments, distances between levels are changed leading to change probability of radiationless processes, i.e., influencing efficiency of quenching (see e.g., [105, 106]). However the course of studying ANS quenching by water has shown that polarity was not able to explain this quenching. A hypothesis has been proposed for the ANS case [107]: a cluster of 3-4 water molecules is formed near ANS amino group, and electron of excited ANS* is transferred to the water cluster. Obviously, this transfer takes place due to H–bonds ANS*–N–H...water. As a result, fluorescence is quenched. H-bond is a bridge for this charge transfer.

Last years, direct data on fluorescence quenching by electron transfer between chromophors and water (or other molecules forming H-bonds) appear (e.g., see [105-110]). In these cases, formation and existence of H-bond are assumed as the cause of the quenching. However, on the other hand, recently some facts were discussed about a probable role of H-bond breaking in the quenching [111]. Indeed, in water as well as in glycerol CAPIDAN has a very “red” absorption spectrum (Table 2). This fact can be a sign of existence H-bonds of the dye carbonyl group(s) =O with OH-groups of these solvents. Excitation spectra have the same “red” peak position as absorption spectra. Coincidence of these excitation and emission peaks could be assumed as a sign of (i) participation of all dye molecules in H-bonds with these solvents, and (ii) participation of all dye molecules in fluorescence emission. These conclusions are supported by the direct data on the fraction of emitting molecules (Table 4): in glycerol all 100 per cent of CAPIDAN molecules participate in the emission.

So, existence of H-bonds solute-solvent in this case does not prevent fluorescence emission. Glycerol heating decreases significantly decay time of this emission (Table 4), i.e., quenches fluorescence; however number of fluorescent molecules stays the same. Heating decreases glycerol viscosity, therefore it can be expected raise of H-bonds breaking. So, this breaking could be assumed as a cause of fluorescence intensity decrease. This hypothesis is supported by some reasons on intramolecular charge transfer at H-bond disappearance [111].

Returning to HSA, this hypothesis can be applied to HSA-bound CAPIDAN. It has the same maximum of excitation spectrum as in water and glycerol (Table 2); unlikely this is an accidental coincidence. Probably, the dye forms H-bond(s) with H-donating groups of the binding site, may be with water which is entrapped by the site. Sites with immobile molecular dynamics, with long-lived H-bonds, give the first dye species with high decay time about 9 ns. Sites with rather mobile interior and short-lived H-bonds give the second and third species with the time of 3 and 1 ns. This conclusion is supported by a direct data on HSA-bound CAPIDAN mobility (see section 7.2): the more the mobility, the less the decay time. In water
phase the H-bonds dye=O…H–OH have very short lifetime, therefore fluorescence decay time and intensity are very low (τ < 0.01 ns).

7. DYE ROTATIONAL MOBILITY IN HETEROGENEOUS ALBUMIN BINDING SITES

Molecular motion in the binding sites has been tested by proton magnetic resonance (\(^1\)H NMR) and by time-resolved polarized fluorescence of the bound dye.

7.1. Proton Magnetic Resonance Data

Free CAPIDAN in water has a narrow \(^1\)H NMR line 3.0 ppm due to 6 protons of dimethylamino group and narrow signals of protons of aromatic rings in the area 7.0–8.4 ppm (Table 6 and Figure 14) [92]. Height of all these lines falls after HSA addition. So, molecular motion around the dye molecule significantly restricted due to its binding with HSA. The amino line 3.0 ppm is well seen on the HSA background signals and therefore is especially sensitive to the binding [92]. Probably, its rotation restricts in HSA molecule; i.e., the group is inside the binding site.

Table 6. CAPIDAN hydrogen atoms responsible for \(^1\)H NMR lines. Deuterium water pD 7.4. Atom numbers are shown in Figure 14. On the basis of [92]

<table>
<thead>
<tr>
<th>Hydrogen atom</th>
<th>37-39, 41-43</th>
<th>34</th>
<th>32</th>
<th>30</th>
<th>26</th>
<th>24</th>
<th>8; 9</th>
<th>7; 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical shift, ppm</td>
<td>3.0</td>
<td>8.1</td>
<td>7.5</td>
<td>8.4</td>
<td>7.0</td>
<td>8.3</td>
<td>7.3</td>
<td>8.0</td>
</tr>
<tr>
<td>singlet/doublet/triplet</td>
<td>s</td>
<td>d</td>
<td>t</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td></td>
</tr>
</tbody>
</table>

Figure 14. CAPIDAN hydrogen atoms responsible for \(^1\)H NMR lines. Chemical shifts (ppm) in deuterium water pD 7.4 are framed. On the basis of [92].
A rather strong Coulomb interaction of the dye COO\(^-\) group with HSA charges (see sections 5 and 8) at physiological ionic strength can signify that the distance between them is rather low, not more than several Angstrom. NMR data shows that the dye amino group on the opposite side of this molecule is hidden of the free water phase too. So, the entire dye molecule is hidden in the HSA binding site (except of COO\(^-\) in the boundary site/water phase).

7.2. Time-Resolved Polarized Fluorescence Data

This approach permits to estimate rotational mobility of each CAPIDAN species separately in HSA binding sites. If a dye solution is irradiated by vertically (index V) polarized light, then its emission is polarized too. At pulse excitation, emission decay \( F(t) \) can be described as a sum of vertically (index VV) and horizontally (index VH) polarized components:

\[
F(t) = F_{VV}(t) + 2F_{VH}(t)
\]  

(20)

Anisotropy of polarized fluorescence, \( r(t) \), decays exponentially in homogeneous solutions \([94, 112, 113]\):

\[
r(t) = \frac{F_{VV}(t) - F_{VH}(t)}{F_{VV}(t) + 2F_{VH}(t)} = r(0) \exp(-t/\psi)
\]  

(21)

where \( r(0) \) is anisotropy at \( t = 0 \) and \( \psi \) is an anisotropy decay time constant (or rotational correlation time). The fluorescence polarized components decay as

\[
F_{VV}(t) = A_v \exp(-t/\tau) + A_v 2r(0) \exp(-t/\gamma)
\]

\[
F_{VH}(t) = A_v \exp(-t/\tau) - A_v r(0) \exp(-t/\gamma)
\]

where \( \gamma = \psi \tau / (\psi + \tau) \)  

(22)

So, these polarized components decay nonexponentially, each of them is a sum of two exponentials. In heterogeneous cases a set of \( m \) decay times \( \tau_i \) and \( \psi_i \) exists. The expressions 22 can be generalized \([112, 113]\) to

\[
F_{VV}(t) = \sum_{i=1}^{m} \left[ A_{vi} \cdot \exp(-t/\tau_i) + A_{vi} 2r_i(0) \cdot \exp(-t/\gamma_i) \right]
\]

\[
F_{VH}(t) = \sum_{i=1}^{m} \left[ A_{vi} \cdot \exp(-t/\tau_i) - A_{vi} r_i(0) \cdot \exp(-t/\gamma_i) \right]
\]  

(23)
e.g., in the case of a minimal heterogeneity \((m = 2)\) each polarized component is a sum of 4 components. Separation of them is not a simple problem \([112, 113]\). In the case of HSA-bound CAPIDAN \(m = 3\), each decay component contains 6 exponentials. Resolution it into 6 components gives a mathematically unstable solution; in other words, a general solution of this problem in analysis of a real experimental data is absent. However recently has been shown \([114]\), that if \(\psi >> \tau\), then each of \(F_{VV}(t)\) , \(F_{VH}(t)\) can be approximated by a single exponential instead of two. Indeed, at \(\psi >> \tau\) parameter \(\gamma = \psi \tau / (\psi + \tau) \approx \tau (1 + \tau / \psi)\) is close to \(\tau\), and two exponentials in Equations 22 (as well as in 23) with almost similar decay times can be assumed as a single one with an intermediate decay time \(\tau_{vi}\) for \(F_{VV}(t)\) or \(\tau_{Hi}\) for \(F_{VH}(t)\).

e.g., in the case of \(\psi = 5\tau\) the error of that approximation is about 1 per cent. So, at \(m = 3\):

\[
F_{VV}(t) \approx \sum_{i=1}^{3} A_i \cdot [1 + 2r_i(0)] \cdot \exp( -t / \tau_{vi} )
\]

\[
F_{VH}(t) \approx \sum_{i=1}^{3} A_i \cdot [1 - r_i(0)] \cdot \exp( -t / \tau_{Hi} )
\]  

(24)

So, decay of \(F_{VV}(t)\) and \(F_{VH}(t)\) should be measured, each of them should approximated by 3 exponentials, this procedure gives 3 pairs of \(\tau_{vi}\) and \(\tau_{Hi}\). Difference between them, i.e., \((\tau_{vi} - \tau_{Hi})\), allows to calculate times of rotational relaxation \(\psi_i\) for each species of the bound dye. This parameter expresses time necessary for deviation of the dye long axis (Figure 16) from its initial direction at excitation moment. If these deviations have an isotropic character, the coefficient of rotational diffusion is equal to

\[
D_{Ri} = k_B T / (8\pi b^3 \eta_i)
\]  

(25)
where \( b \) is half of the dye size in the direction along the long axis, \( \eta_i \) is environment viscosity for this species, \( k_B \) is Boltzmann constant and \( T \) is temperature.

In the case of CAPIDAN bound to fatty acid free HSA the species 1 has \( r(0) \) close to 0.30 and \( (\tau_{V1} - \tau_{H1}) = (8.23 - 8.79) = -0.56 \) ns; decay of its polarized components can see in Figure 15. Data of the bound dye species mobility are presented in Table 7.

As has been proposed above, one (carboxylic) end of the dye molecule is tightly bound with a positive charge(s) of the binding site (see sections 5 and 8). But a rotational freedom exists. The first species has \( \psi \) equal or more than an estimate for rotation of HSA globule (40–80 ns). So, this species is really immobile in its binding site. Probably, its motion is strongly limited. The second and especially third species move rather free.

### Table 7. Rotational mobility, \( \psi_i \) (ns), of HSA-bound CAPIDAN in isolated HSA (fatty acid free) without or with palmitate addition and in serum of a healthy donor

<table>
<thead>
<tr>
<th>Sample</th>
<th>Species</th>
<th>1 “9 ns”</th>
<th>2 “3 ns”</th>
<th>3 “1 ns”</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA fatty-acid free</td>
<td></td>
<td>72 ± 7</td>
<td>41 ± 8</td>
<td>9.6 ± 1.3</td>
</tr>
<tr>
<td>HSA + palmitate = 1: 0.8 (mol/mol)</td>
<td></td>
<td>45 ± 5</td>
<td>15 ± 5</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td>49 ± 4</td>
<td>21 ± 3</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

As has been proposed above, one (carboxylic) end of the dye molecule is tightly bound with a positive charge(s) of the binding site (see sections 5 and 8). But a rotational freedom exists. Therefore it can be suggested that other end of the dye molecule is relatively free for movement. Then this movement is probably like to wobbling of the amino-end (Figure 16). That wobbling of the second and third species is a probable cause of fluorescence depolarization. This free motion could facilitate fluorescence quenching by water and, as a consequence, decrease of the dye decay times \( \tau_2 \) and \( \tau_3 \) up to 3 and 1 ns, respectively. In the

---

Figure 16. Hypothetical scheme of CAPIDAN wobbling in HSA binding site.
sites for the first species so fast motion is absent, therefore \( \tau \) has its maximal value. Wobbling of other aromatic ligands bound to HSA has been supposed earlier (e.g., [115]).

It is interesting that this motion in serum is faster than in isolated, fatty acid free HSA (Table 7). Perhaps, this is an effect of fatty acids: e.g., palmitate addition to fatty acid free HSA leads to decrease \( \psi \) (i.e., raise of motion) up to level of HSA in serum (Table 7). Palmitate addition in this sample was equal to a mean level of fatty acids in natural sera of healthy donors. This motion is almost nonsensitive to pH changes from 4 to 9; so, N-F and N-B conformational transitions do not influence freedom of the dye rotational motion in HSA.

8. CHLORIDE/NITRATE SUBSTITUTION FOR STUDY OF DRUG-PROTEIN COULOMB INTERACTION

CAPIDAN binding to HSA depends on ionic strength, \( I \), of solution. Initial fluorescence intensity, \( F_0 \), at low \( I \) decreases at KCl addition, i.e., at \( I \) raise. Effect of this \( F_0 \) decrease to \( F \) can be expressed as \( (F_0 - F) / F_0 \). Ionic strength is proportional to square root of KCl concentration in accordance with the Debye-Huckel theory. Indeed, in the case of KCl there is a dependency of this effect on \( I^{0.5} \) rather close to straight line (Figure 17). There exist similar examples of other dyes which interaction with HSA is sensitive to ionic strength and is described as a linear function of \( I^{0.5} \) (e.g., [115]) though the binding dye-protein should be described by a much more complicated function.

All amplitudes of the three CAPIDAN species lower due to \( I \) raise, i.e., concentrations of bound dye of all species fall (Table 9) [116, 117]. The most sensitive to ionic strength is the first species: the raise \( I \) from 0.02 to 0.52 M causes 3-fold decrease of \( r_1 \) (Table 9 and [116]), and from 0.002 M to 0.52 M – by 5.1-fold [92]. If it is assumed that the ionic strength prevents Coulomb interaction of the dye negative charge with HSA positive charges, then the free energy \( \Delta G \) of the interaction can be estimated [92]. This free energy at \( I = 0.002 \) M is equal to \( \Delta G_{0.002} \) and at 0.52 M – to \( \Delta G_{0.52} \). It can be assumed that \( | \Delta G_{0.002} | >> | \Delta G_{0.52} | \) in accordance with Debye-Huckel theory. So, a transition from \( I = 0.52 \) M to \( I = 0.002 \) M for the first species gives a change of the free energy of dye-site interaction:

\[
\Delta G_{0.002} \approx \Delta G_{0.002} - \Delta G_{0.52} \approx -RT \cdot \ln(5.1) = -1 \text{kcal/mol}
\] (26)

On the basis of the data presented in Figure 17, it could be estimated that the Coulomb energy of the first species lowers to –0.4 kcal/mol at physiological ionic strength [92]. The similar interaction takes place in the cases of the second and third species but their energy is lower; may be, in these cases distances between charges dye/albumin are larger (this hypothesis is supported by facts of more rotational mobility, see section 7.2 and Figure 16). These estimations are rather approximative.

On the other hand, if chloride anion is replaced by nitrate anion, then fluorescence decrease is nonlinear function of \( I^{0.5} \) and rather stronger than in the case of chloride (Figure 17). So, nitrate causes by (i) raising ionic strength but (ii) has an additional, specific ability to decrease of HSA-CAPIDAN fluorescence. Either nitrate decreases its decay time \( \tau \), or it suppresses dye binding. However \( \tau \) of all three species stays almost unchanged at nitrate addition. Titration of HSA by the dye (Figure 18) has shown that the intercept on the vertical
axis is the same for both anions. So, number of binding sites is unchanged at replacing chloride by nitrate. On the other hand, the intercept on the horizontal axis is decreased under this replacement. This intercept is an estimation for the binding constant. So, nitrate suppresses the binding constant $K_1$, e.g., by 2.9 times at this partial replacement of chloride by nitrate. $K_2$ is 1.8-fold decreased under the same conditions, $K_3$ is decreased too.

Thus, a specific effect of nitrate as compared with chloride is suppressing the dye binding to HSA by decreasing the binding constant. What is the molecular basis of that effect? It can be assumed that nitrate suppresses the Coulomb interaction of the dye negative charge and positive charges on the interface of the site I and water phase, i.e., HSA lysine and arginine residues (Figure 19). Either nitrate anion specifically binds to lysine neutralizing its positive charge or it neutralizes the arginine charge. Conductometric experiments on charge interaction in electrolyte solutions have shown [116, 117] that the nitrate anion has significantly more affinity to guanidine cationic group of arginine than chloride anion.

![Graph showing decrease of CAPIDAN fluorescence intensity due to KCl or KNO₃ addition to solution of CAPIDAN and HSA in 0.01 M phosphate pH 7.4. HSA was fatty acid free.](image)

Figure 17. Decrease of CAPIDAN fluorescence intensity due to KCl or KNO₃ addition to solution of CAPIDAN and HSA in 0.01 M phosphate pH 7.4. HSA was fatty acid free.

So, it can be suggested that the negatively charged group $-\text{COO}^-$ of CAPIDAN interacts with an arginine guanidine group on the interface of the site I/water phase. This arginine could be either Arg 218, Arg 222 or Arg 257 (Figure 19). Binding of another anionic drug, warfarin, to the same site significantly enhances after Lys199 replacement by an uncharged residue but falls threefold after Arg218 replacement [102, 118]. These data support the suggestion that CAPIDAN also interacts with arginines of the site I, may be with Arg 218, and nitrate anions hinder this interaction.

That technique of nitrate/chloride replacement can be used for detecting the role of Coulomb interaction arginine-ligand in ligand binding by proteins.
Table 9. Influence of ionic strength \( I \) on CAPIDAN binding to HSA. \( r_1 \), \( r_2 \) and \( r_3 \) are concentrations of the dye species bound to HSA; \( r_i / r_i(0.02) \) – these concentrations are normalized to their values at \( I = 0.02 \) M. Fatty acid free HSA, pH 7.4. Data from [116]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ionic strength ( I ), mol/Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>( r_1 / r_1(0.02) )</td>
<td>1.00</td>
</tr>
<tr>
<td>( r_2 / r_2(0.02) )</td>
<td>1.00</td>
</tr>
<tr>
<td>( r_3 / r_3(0.02) )</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Figure 18. Titration of HSA by CAPIDAN in the presence of chloride 0.63 mol/litre and after partial equimolar substitution of 0.38 mol/litre chloride by nitrate. \( A_1 \) is amplitude of the CAPIDAN first species, \( A_{1\text{max}} \) is its maximal value. \( K_1 \) is the binding constant. Fatty acid free HSA, pH 7.4. On the basis of the data from [117].

Figure 19. Schematic representation of positive charges on the surface of the Site I in HSA crystals. On the basis of the crystallographic data of [102, 119, 120]. K is lysine, R is arginine, W is tryptophan.
9. THERMODYNAMICS OF CAPIDAN-ALBUMIN INTERACTION

The fact of CAPIDAN binding to HSA signifies in the formal language of physics that free energy of this solution decreases as a result of the complex formation. This decrease is $\Delta G$. The simplest formalism for HSA-dye interaction could be as follows. Two processes take place: (i) association of the dye with the HSA binding site and (ii) dissociation of their reversible complex. Rate constants of these processes are $k_{\text{Ass}}$ and $k_{\text{Dis}}$ (Figure 20). To form such complex, it needs to overcome a barrier $\Delta G_A$, its dissociation needs $\Delta G_D$ (Figure 20). If the difference $\Delta G = (\Delta G_A - \Delta G_D) < 0$, then the complex is thermodynamically probable.

The binding constant (see Equation 16) $K_B = k_{\text{Ass}} / k_{\text{Dis}}$, and the rate constants can be assumed as exponential functions of $\Delta G_A$, $\Delta G_D$. The free energy includes two components: enthalpy $\Delta H$ and entropy $\Delta S$. So,

$$K_B = \frac{k_{\text{Ass}}}{k_{\text{Dis}}} \frac{k_{d0}^{\text{Ass}} \exp(-\Delta G_A / RT)}{k_{d0}^{\text{Dis}} \exp(-\Delta G_D / RT)} = \frac{k_{d0}^{\text{Ass}} \exp(-\Delta H_A - T\Delta S_A / RT)}{k_{d0}^{\text{Dis}} \exp(-\Delta H_D - T\Delta S_D / RT)}$$

(27)

Then

$$\ln K_B = [(\ln \frac{k_{d0}^{\text{Ass}}}{k_{d0}^{\text{Dis}}}) + \frac{\Delta S_B}{R}] - \frac{\Delta H_B}{RT}$$

(28)

This equation shows that measuring $K_B$ at different temperatures $T$ can help calculate enthalpy of the binding, i.e., $\Delta H_B$. However estimation of entropy, $\Delta S_B$, is not so simple because the temperature-independent part of the Equation 28 includes a ratio $k_{A0}/k_{D0}$ besides of entropy, and there is no a simple way to estimate this ratio.

Figure 20. Scheme of energetic barriers at HSA-CAPIDAN complex formation.
Table 10. Enthalpy, Δ\(H_B\), of binding CAPIDAN to HSA. Data of a formal calculation on the basis of Equation 28 for the temperature interval from 15 to 45 °C. Fatty acid free HSA. pH 7.4. Mean error ± 0.2 kcal/mol

<table>
<thead>
<tr>
<th>Species</th>
<th>Ionic Strength, M</th>
<th>Δ(H_B), kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02</td>
<td>0.15</td>
</tr>
<tr>
<td>1</td>
<td>0.16</td>
<td>-1.3</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>-3.6</td>
</tr>
<tr>
<td>2</td>
<td>0.16</td>
<td>-1.4</td>
</tr>
<tr>
<td>3</td>
<td>0.16</td>
<td>-0.7</td>
</tr>
</tbody>
</table>

In the case of CAPIDAN-HSA interaction, both parameters of fluorescence decay of each species, \(A_i\) and \(\tau_i\), are dependent on temperature. The first of them, \(A_i\), reflects the concentration of bound dye, while the second, \(\tau_i\), depends on quenching processes in the binding site. Formally calculated values of enthalpy, Δ\(H_B\), of CAPIDAN binding to HSA are shown in Table 10.

Figure 21. Dependency of the amplitude \(A_1\) of the CAPIDAN first species on temperature \(T\) at three ionic strengths \(I\). Fatty acid free HSA, pH 7.4.

Dependency of \(A_1\) on \(T\) at different ionic strength is shown in Figure 21. As can be seen, the upper curve (low ionic strength) has a complex form. At 15-48 °C there are not significant \(A_1\) changes. So, here the binding of the first species is independent of \(T\). This fact formally could mean that the binding enthalpy, Δ\(H_B\), is close to zero (Table 10). However the binding exists, therefore

\[
\Delta G_B = \Delta H_B - T \Delta S_B < 0; \text{ then } \Delta H_B \approx 0; \Delta S_B > 0
\] (29)
i.e., the driving force of the binding could be a raise of entropy: \( T \Delta S_B > 0 \). In this temperature interval raising \( I \) leads to increasing of the slope of the curves in Figure 21, i.e., binding at physiological \( I \) and especially at high \( I \) is decreased at heating. So, formally the value of enthalpy \( \Delta H_B \) grows at ionic strength increasing.

These results seem paradoxical. All experiments show existence of Coulomb interaction the dye-HSA which is suppressed by ionic strength (see above, section 8). This tendency is seen in Figure 21: amplitudes decrease at ionic strength increase. At the same time presence of the Coulomb force should be expressed by rather high enthalpy which should decrease up to \( \approx 0 \) at high \( I \). However results of Figure 21, as it seems, are opposite.

To try solving this paradox, the next hypothesis can be proposed. Enthalpy \( \Delta H_B \) can be calculated using Equation 27 if its parameters, i.e., \( k_{Ass}/k_{Dis} \), \( \Delta H_B \), \( \Delta S_B \) are temperature-independent constants. However the binding site I has a rather complex structure, and these parameters could be interdependent or/and temperature-dependent. The first explanation of the horizontality of the 15-48 °C interval on the upper curve, i.e., \( \Delta H_B = 0 \), seems unlikely (see above). The second formal explanation is an enthalpy raise at heating:

\[ \Delta H_B \approx -hT \quad \text{were} \quad h>0 \quad \text{is a constant.} \]  

(30)

The interval of 48-55 °C of the upper curve supports in general this hypothesis: the more \( T \), the more slope of the curve, i.e., formally the more \( \Delta H_B \). The third case may be a raise of the ratio \( k_{Ass}/k_{Dis} \) or entropy \( \Delta S_B \) at heating.

Such clear deviations from the simplest model (Equation 27) occur only at low ionic strength. May be, these anomalies are due to mutual repulsion of positive charges in their cluster on the interface Site I–Water phase (Figure 19). This repulsion enhances at low ionic strength and leads to swelling of the cluster: charges move apart, the space is filled up by water. The anomalies at low ionic strength are an effect of the dye negative charge and temperature on this swelling of the positively charged cluster.

### 10. FATTY ACID INFLUENCE ON THE DRUG-BINDING SITES

Transfer of long-chain nonesterified fatty acids (FA) by HSA is one of the main HSA functions. FA concentration in plasma is a risk-factor for sudden cardiac death [121, 122]. Electron paramagnetic resonance signals of spin-labeled FA were significantly changed at cancer and other diseases [123, 124]. FA content of plasma depends on motor activity of rats at stress [125] etc. So, FA level is tightly associated with physiological, emotional and pathological status.

Plasma FA are mainly bound to HSA. Mean ratio FA/HSA in blood is close to 0.7–1 mol/mol [36] but HSA molecule is able to bind even 10-12 FA molecules. One site for FA binding has the binding constant about \( 10^7 \) M\(^{-1} \), two sites have \( 10^6 \) M\(^{-1} \) and 5 sites have near \( 10^3 - 10^4 \) M\(^{-1} \) [36]. Localization of sites for the first six FA molecules (i.e., sites of the most binding constant) in HSA crystals is shown in Figure 12 [126, 127].

FA influence binding of fluorescent [128-131] and spin [132] probes and other ligands to HSA. Binding of some ligands is suppressed while of others is enhanced by FA addition. CAPIDAN fluorescence enhances due to addition of 1-5 FA molecules per one HSA
molecule [23, 133]. Enhancement of the total fluorescence may be a result of decay time or/and amplitude changes. In the case of FA addition to HSA all three decay times of the three decay CAPIDAN species are slightly lower while total fluorescence intensity increases significantly due to raise of amplitudes; i.e., FA increase binding of the dye to HSA. However sensitivity of the three main species of the probe to FA is different: the first one has a minimal sensitivity and the third one has maximal (Figure 22).

What is a mechanism of this binding raise? FA localization in HSA crystals is shown in Figure 12 (see section 5). Negatively charged carboxyl of the FA molecule number 2 localizes near positively charged Arg 257 in the site I [119]. It seems that in water solutions the negatively charged FA carboxyl should be attracted to the positively charged cluster on the surface of the site I (see Figure 19 in section 8). As a result, the binding constant for this negatively charged dye is expected to be decreased after FA appearing. But it raises. Moreover, the first species is the most sensitive to the charge of the cluster (see section 8), however it is almost insensitive to FA (Figure 22). The third species, in contrast, is almost insensitive to ionic strength but its population increases by 3 times at addition of 3 FA molecules. FA effects on the dye binding are almost independent of ionic strength of solution; this is an indirect evidence of absence Coulomb interaction of FA and the dye in HSA.

So, the FA negatively charged carboxyl unlikely localizes in the positive cluster of the site I in water solutions. There are no evidences of competition of the dye and the first three FA molecules. Quite the contrary, these FA molecules enhance the dye binding to HSA. Physical chemical causes of this situation need to be clarified.

Figure 22. Influence of palmitate addition to HSA on amplitudes of three species of CAPIDAN fluorescence decay. Initial HSA was fatty-acid free. pH 7.4, ionic strength 0.16 M.
CONCLUSION

A lot of aromatic substances, both fluorescent and nonfluorescent, have been used to study HSA drug binding sites. CAPIDAN is one of those substances. After its addition to diluted plasma or serum the main part of its fluorescence, 90–95 per cent, originates from its complexes with HSA, in contrast with many other aromatic substances. This property leads to study of HSA binding sites in their intact state without any extraction and fractionation procedures. Therefore this dye has been used to detect changes of the binding sites which accompany some pathological processes. In some cases this information is useful for clinical diagnostics exceeding routine clinical analyses. The sensitivity to site changes is raised by using time-resolved measurements of fluorescence decay and three new methods of the data analysis: (1) estimation of absolute values of concentration of fluorescent species in heterogeneous biological objects; (2) measurement of dye rotational mobility in heterogeneous albumin binding sites; and (3) a method of chloride/nitrate substitution to detect interaction of a drug negative charge with arginine positive charges in the albumin binding sites.

A part of disease-accompanied changes of HSA molecule can be attributed to variations of quantity and content of HSA-bound nonesterified fatty acids; however these variations can not explain in full measure the structural changes of the binding sites detected by CAPIDAN.

REFERENCES


