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# STUDY ON THE INTERACTION OF 2-CARBOXYPHENOXATHIIN WITH BOVINE SERUM ALBUMIN AND HUMAN SERUM ALBUMIN BY FLUORESCENCE SPECTROSCOPY AND CIRCULAR DICHROISM

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The interaction between 2-carboxyphenoxathiin and bovine serum albumin (BSA)/ human serum albumin (HSA) has been studied by fluorescence spectroscopy and circular dichroism. The binding of 2-carboxyphenoxathiin quenches the BSA and HSA fluorescence. By the fluorescence quenching results, it was found that the binding constant $K$=3.2*10$^5$/1.09*10$^5$ M$^{-1}$, and number of binding sites $n$ = 0.95/0.82. The distance, $r$, between donor (BSA or HSA) and acceptor. (2-carboxyphenoxathiin) was obtained according to the Förster's theory of non-radiatioactive energy transfer. The interaction between 2-carboxyphenoxathiin and BSA/HSA has been verified as consistent with the static quenching procedure and the quenching mechanism is related to the energy transfer. Circular Dichroism results revealed that the binding of 2-carboxyphenoxathiin to BSA/HSA do not induce conformational changes in albumins.

## INTRODUCTION

Serum albumins are abundantly found in blood plasma and are often termed transport proteins.[1-4] They are circulated in the body several times and act as carriers for numerous exogenous and endogenous compounds. The most popularly studied albumins are bovine serum albumin (BSA) and human serum albumin (HSA). Both BSA and HSA have very high conformational adaptability to a great variety of ligands.[4] BSA and HSA mainly differ in tryptophan content. Except for this, the amino acid compositions of the two proteins are nearly the same. HSA has one tryptophan group at the 214 position, while BSA has tryptophan groups at 134 and 212 position. Primarily, three domains and six principal binding sites have been identified for several important biomolecules.[4-6]

The interaction of the aromatic or/and heteroaromatic carboxylic acids with proteins was often investigated.[7, 8] The main interest was determined by the pH dependence of the spectral properties correlated with the acid-base equilibrium, allowing for using them in aqueous media.

In this paper, we continue our preceding studies[9] on the interaction of phenoxathiin carboxylic acids with bovine and human serum albumins (BSA and HSA).

The first derivative used, 3-carboxyphenoxathiin, shows a significant interaction with both albumins (article in press). The experiments were performed by monitoring the quenching of the albumin intrinsic fluorescence in the presence of increasing amounts of 3-carboxyphenoxathiin. The experimental data allow for the determination of the binding constants and show that the Forster energy transfer is the probable quenching mechanism. Circular dichroism measurements evidence changes of the protein ellipticity correlated to modifications in the secondary protein structure upon the interaction process. In the followings, we use another derivative, 2-carboxyphenoxathiin, **I** (Fig. 1). Generally, the 2-substituted derivatives on the phenoxathiin ring are characterized by low fluorescence quantum yields.[10] However, from some preliminary experiments we expect that 2-carboxyphenoxathiin can also be used for the same purpose.

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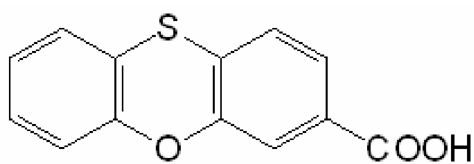
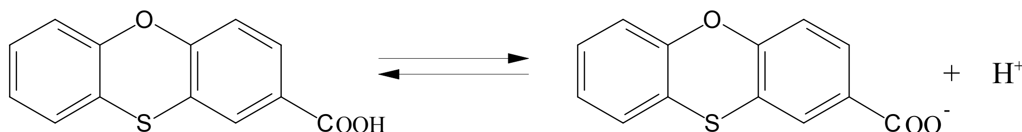



Fig. 1 – The structure of 2-carboxyphenoxathiin, **I**.

The $pK_a$ of the acid-base equilibrium of **I** (Scheme 1) is 4.57. Therefore, at pH 7.4 we assume that the predominant form is the dissociated one.




Scheme 1

## RESULTS AND DISCUSSION

### 1. Stern-Volmer plots

The increase in the ligand concentration determines a quenching of the intrinsic fluorescence of both proteins. An example is given in **Fig. 2** for the system BSA-**I**. The representative Stern-Volmer plots are displayed in the inset of **Fig. 2**. It can be seen that for both albumins, the plots present a negative deviation from the linearity.

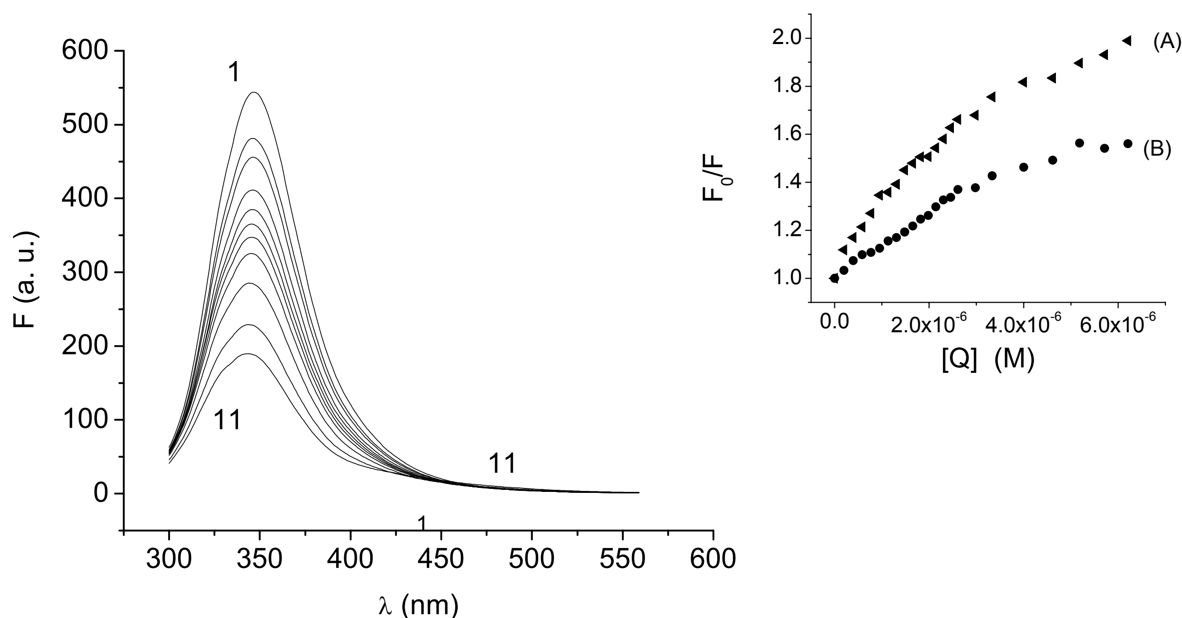



Fig. 2 – Fluorescence spectra of the system BSA – **I** at increasing concentration of **I**.
[BSA]= $3*10^{-6}$ M; 1-11: d/p =1-3; $\lambda_{ex}$ =286 nm.
Inset: Stern Volmer plots: (A) **I**-BSA; (B) **I**-HSA.

In order to determine the SV constant, $K_{SV}$, we used the modified SV equation[11] characteristic to the cases in which the fluorophore is not totally available to the quencher:

$$\frac{F_0}{F_0 - F} = \frac{1}{f} + \frac{1}{f * K_{SV} * [Q]} \qquad (1)$$

in eq. (1), $F_0$ and F represent the fluorescence in the absence and presence of a quencher, respectively, [Q] is the quencher concentration and f is the fraction of the fluorophore accessible to the quencher. The plots are presented in **Fig. 3**, for a d/p (drug to protein ratio) range 0.26-3.

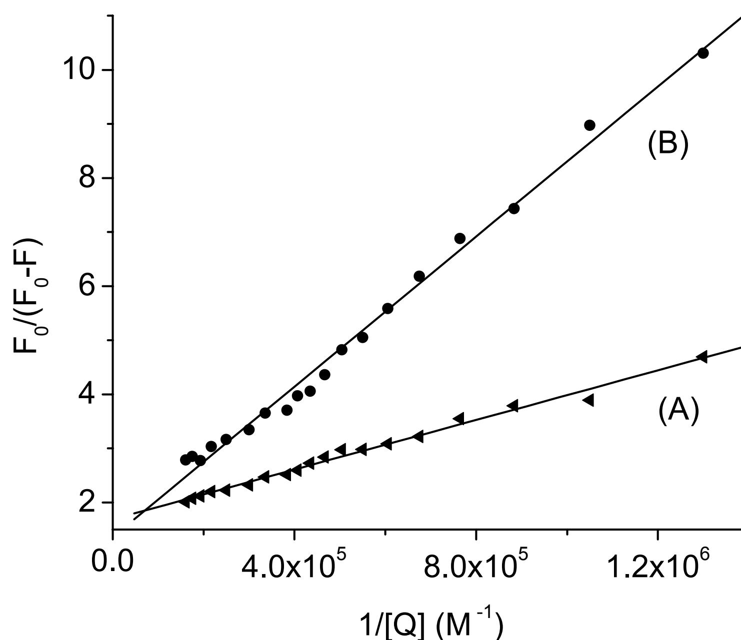



Fig. 3 – Modified Stern-Volmer plots: (A) **I**-BSA; (B) **I**-HSA.
The experimental points were fitted to eq. 1.

Taking into account the lifetime of albumins of $10^{-8}$ s and an averaged SV constant of about $10^5$ $M^{-1}$ we obtain for the rate constant of the bimolecular quenching process, a value of $10^{13}$ $M^{-1}$ $s^{-1}$ which largely overrides the accepted limit of the rate constant of the diffusional quenching implying biopolymers, $2\times10^{10}$ $M^{-1}$ $s^{-1}$. This observation supports the fact that, the experimental quenching of albumin fluorescence is due to a static process rather than to a dynamical one. The constant for the static quenching was obtained by the Lineweaver-Burk equation: [12]

$$(F_0\text{-}F)^{-1} = F_0^{-1} + K^{-1}F_0^{-1}\,[Q]^{-1} \qquad (2)$$

The Lineweaver – Burk (LB) plots are presented in **Fig. 4** and the K values are also listed in **Table 1**.

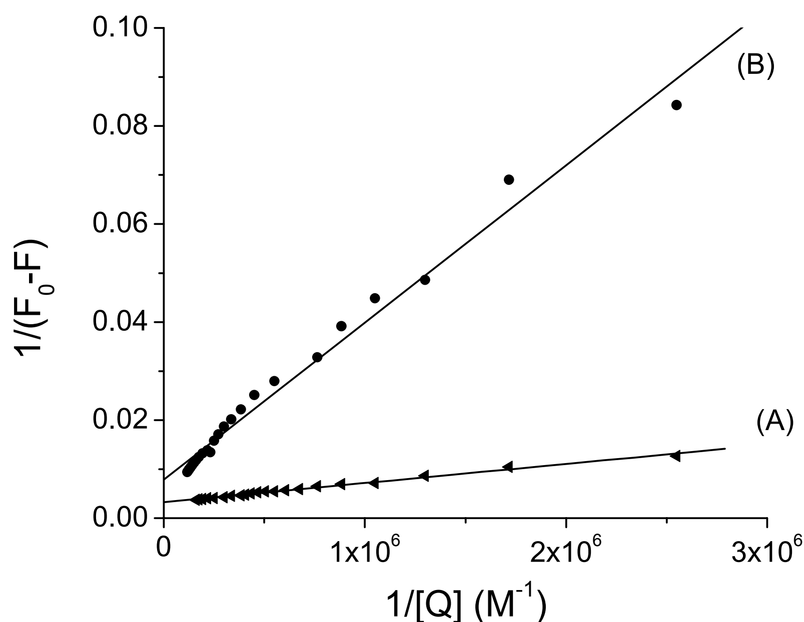



Fig. 4 – LB plots: (A) **I**-BSA; (B) **I**-HSA. d/p=0.26-3; [BSA] = [HSA] = $3*10^{-6}$ M.

*Table 1*

Binding constants obtained by Lineweaver-Burk (LB) equation

| Albumin | LB | |
|---|---|---|
| | $K(M^{-1})$ | r |
| HSA | $2.43*10^5$ | 0.994 |
| BSA | $8.37*10^5$ | 0.995 |

**2. Determination of the binding parameters**

Starting with the Scatchard equation[7] for a single class of *n* independent binding sites, eq. (3):

$$\nu = \frac{n*K*[L_f]}{(1+K*[L_f])} \quad (3)$$

several models are given in literature for the determination of the binding parameters, the number of sites (n) and the binding constants (K).[14-16]

In eq. (3), ν represents the binding ratio, the ratio of the bound ligand to the total protein, $[L_b]$ / $[P_t]$, and $[L_f]$ is the concentration of the free ligand at equilibrium. The calculation of ν is made considering that the measured fluorescence, F, at a given ligand concentration, is due to the unbound protein, [P]:

$$\frac{F_0}{F} = \frac{[P_t]}{[P]} \quad (4)$$

$$\nu = \frac{[L_b]}{[P_t]} = \frac{([L_t]-[L_f])}{[P_t]} = n*\frac{([P_t]-[P])}{[P_t]} = n*\frac{(F_0-F)}{F_0} \quad (5)$$

where $[L_t]$, $[P_t]$ are the total (analytical) concentrations of the ligand and the albumin, respectively.

The main problem of the corresponding equations is the replacement of the free ligand concentration by the total concentration, i.e. by the amount of the added ligand, $[L_{free}] = [L_{total}]$, approximation that is not always valid. In the following, in order to rationalize our experimental data on the albumin-2-carboxyphenoxathiin derivative, we have focused on some of these equations in which such approximations are avoided. The fluorescence experimental data were fitted to the following linear equation.

$$\log\frac{F_0-F}{F} = n\times\log K - n\times\log\frac{1}{[L_t]-\frac{F_0-F}{F_0}\times[P_t]} \quad (6)$$

The slope of the linear plot of log $(F_0\text{-}F)/F$ vs. log $(1/([L_t] -(F_0\text{-}F)\times[P_t]/F_0))$, eq. (6), gives the number of sites and the intercept with the ordinate, the product n*log K. The plots for the two proteins are displayed in **Fig. 5** and the binding parameters are presented in **Table 2**. In order to better evidence the scattering of the experimental data, the results for a duplicate experiments (number of points = 60) are displayed in the inset of Fig. 5. The averaged values are listed in **Table 2**.

The averaged value of the number of sites, n, is about 0.78-0.95 reflecting a 1:1 interaction with a significant binding constant about $10^5$ M for both albumins.

*Table 2*

Binding parameters obtained by eq. (6)

| Albumin | Eq. 6 | |
|---|---|---|
| | n | $K(M^{-1})$ |
| HSA | 0.82 | $1.09*10^5$ |
| BSA | 0.95 | $3.20*10^5$ |

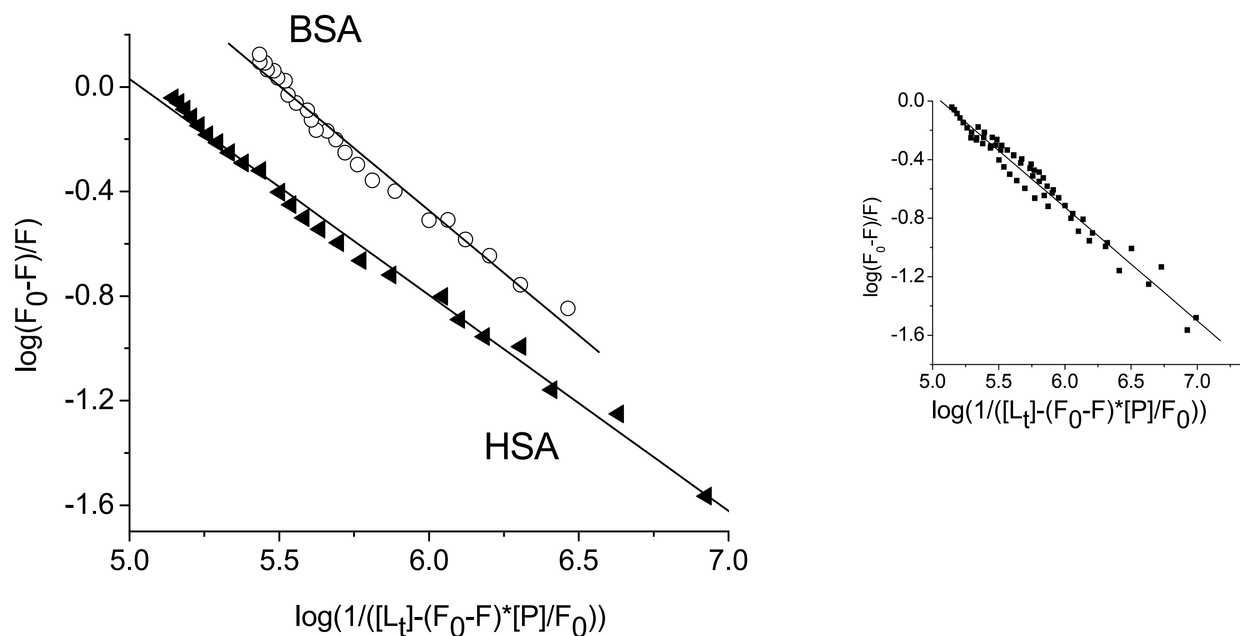



Fig. 5 – Experimental data for the albumin-I systems fitted to eq. (6): **I**-BSA (r = 0.991); **I**-HSA (r = 0.996). Inset: a duplicate experiment for the system **I**-HSA (r = 0.983).

### 3. Synchronous fluorescence

Synchronous fluorescence is usually used to identify the change upon interaction around the tryptophan and tyrosine residues responsible to the protein fluorescence. The fluorescence spectra are recorded under the condition $\Delta\lambda = \lambda_{ex} - \lambda_F$ = constant with the specific values $\Delta\lambda$ = 15 nm for Tyr and $\Delta\lambda$ = 60 nm for Trp.[17]

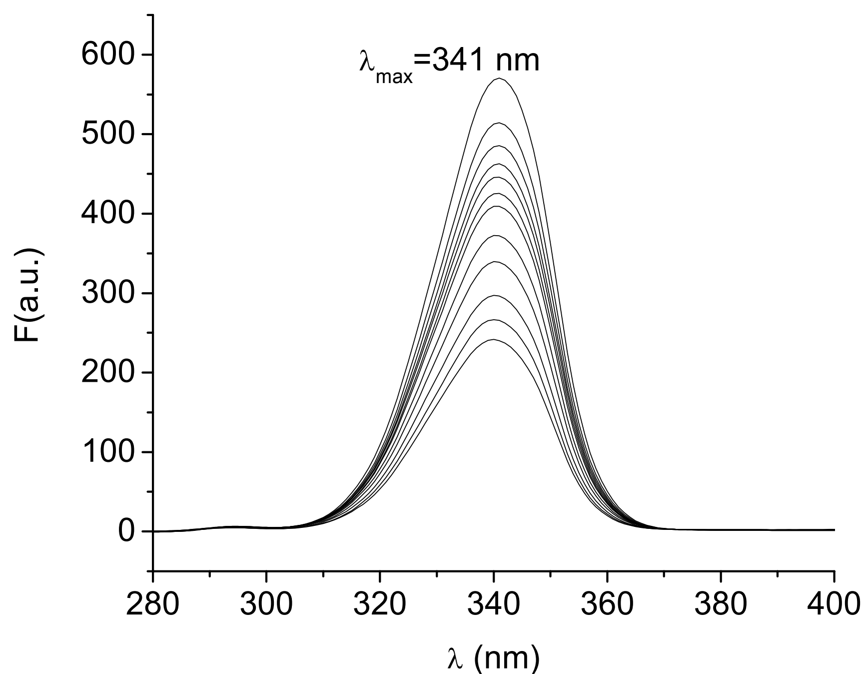



Fig. 6 – Synchronous fluorescence spectra of BSA in the presence of increasing amounts of 2-carboxyphenoxathiin. $\Delta\lambda$ = 60 nm; [BSA] = $3*10^{-6}$ M.

A bathochromic shift of $\lambda_{max}$ at increasing concentration of the drug, implies that the amino acid residues are in a polar environment, and are more exposed to the solvent.[18-21] A hypsochromic shift of the band denotes a reversal effect, an increase of the local hydrophobicity. In our case recording the synchronous fluorescence spectra using $\Delta\lambda$ = 60 nm (**Fig. 6**) does not evidence a shift of the fluorescence maximum, but the overall shape of the band is somewhat modified, a shoulder being apparent at 347 nm. The same lack of a visible effect was also noticed making the measurements with $\Delta\lambda$ = 15 nm. The synchronous fluorescence spectra show that the environment of the Trp-214 (HSA) or Trp-212 (BSA) residues is practically not changed by the interaction with the drug.

## 4. Energy transfer between 2-carboxyphenoxathiin and bovine serum albumin (BSA) and human serum albumin (HSA)

The fluorescence resonance energy transfer (FRET) represents one of the quenching mechanisms and occurs whenever the emission spectrum of the fluorophore (donor) overlaps with the absorption spectrum of the acceptor.

The overlap of the UV absorption spectrum of 2-carboxyphenoxathiin with the fluorescence emission spectrum of HSA is shown in **Fig. 7**. The distance between the donor and acceptor can be calculated according to Förster's theory.[22]

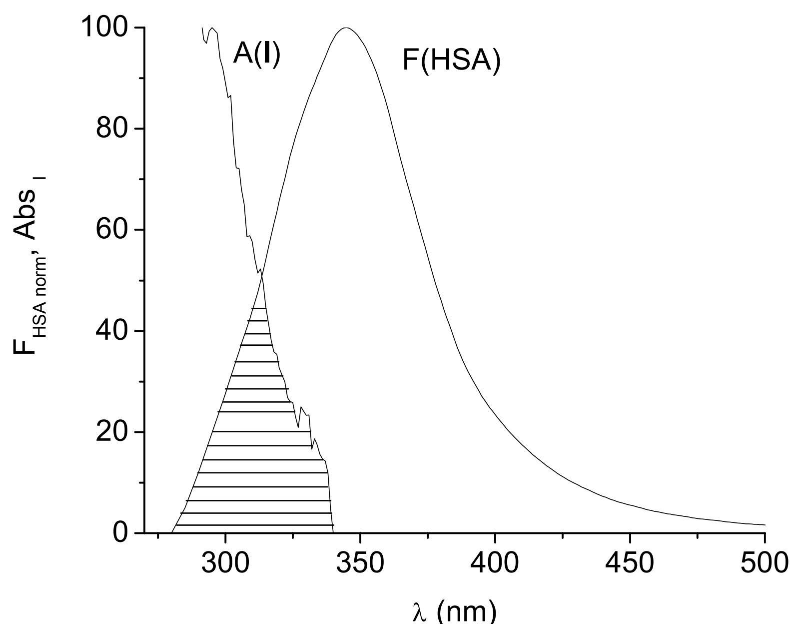



Fig. 7 – Overlap of the normalized absorption (**I**) and fluorescence spectra (HSA).

The efficiency of energy transfer, *E*, is calculated using equation (7):

$$E = 1- \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \qquad (7)$$

where $F_0$ and $F$ are the fluorescence intensities of BSA / HSA measured in the absence of the ligand and at equal protein–ligand concentration, respectively,[23,24] $r$, the distance between acceptor and donor and $R_0$ is the critical distance when the transfer efficiency is 50%.

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \qquad (8)$$

where $k^2$ is the spatial orientation factor of the dipole; $N$, the refractive index of the medium; $\Phi$, the fluorescence quantum yield of the donor; and $J$ is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. $J$ is given by:

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \sum F(\lambda) \Delta\lambda \qquad (9)$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength $\lambda$ and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength $\lambda$. According to literature data,[25] $K^2$ = 2/3, $N$ = 1.36 and $\Phi$ = 0.15. From eqs. (7) – (9), $J$, $R_0$ (nm), $E$ and $r$ (nm) were calculated and are listed in **Table 3**.

*Table 3*

Energy transfer parameters: the overlap integral (J), the efficiency (E), the critical distance ($R_0$) and the donor acceptor distance (r).

| Albumin | J ($cm^3$ l $mol^{-1}$) | E | $R_0$ (nm) | r (nm) |
|---|---|---|---|---|
| BSA | $1.30 \times 10^{-16}$ | 0.424 | 1.085 | 1.141 |
| HSA | $8.26 \times 10^{-16}$ | 0.278 | 1.128 | 1.322 |

The donor-to-acceptor distance, $r < 7$ nm, $0.5R_0 < r < 1.5R_0$,[26-28] indicated that the energy transfer from BSA / HSA to 3-carboxyphenoxathiin occurs with high possibility.

## 5. Circular dichroism spectra

The serum albumins present two negative bands in the UV range, located at 208 nm and 222 nm characteristic for the α-helical structure. [27,30,31] Increasing the ligand concentration, the CD spectrum of the protein is modified, a decrease in the intensities of both bands being noticed (**Fig. 8)**.

As the measured ellipticity depends on the ligand concentration, the CD spectroscopy can also be used to determine the binding parameters[29] but the errors are usually larger than by the fluorescence method. In our case, we have adapted eq. 6, to be used for the estimation of the binding parameters using the measured ellipticities in the presence of increasing the ligand concentration. The plot for the system **I**-HSA is presented in **Fig. 9**. Although the experimental points are more scattered than in the case of fluorescence measurements, the binding parameters, $K=1.58*10^5$ $M^{-1}$ and n=1.2 are similar with that previously obtained by the former spectral method. For the second system, **I**-BSA, the results were less reliable, the binding constant being significantly lower than that obtained by the fluorescence method.

The main information provided by the CD method concerns the changes in the protein secondary structure upon drug (ligand)-protein interaction. The CD data were expressed in terms of mean residue ellipticity (MRE) in deg $cm^2$ $dmol^{-1}$, according to the following equation:

$$\text{MRE} = \frac{ObservedCD(m\deg)}{C_p nl \times 10} \quad (10)$$

where $C_p$ is the molar concentration of the protein, $n$ is the number of amino acid residues of the protein and $l$ is the path length.

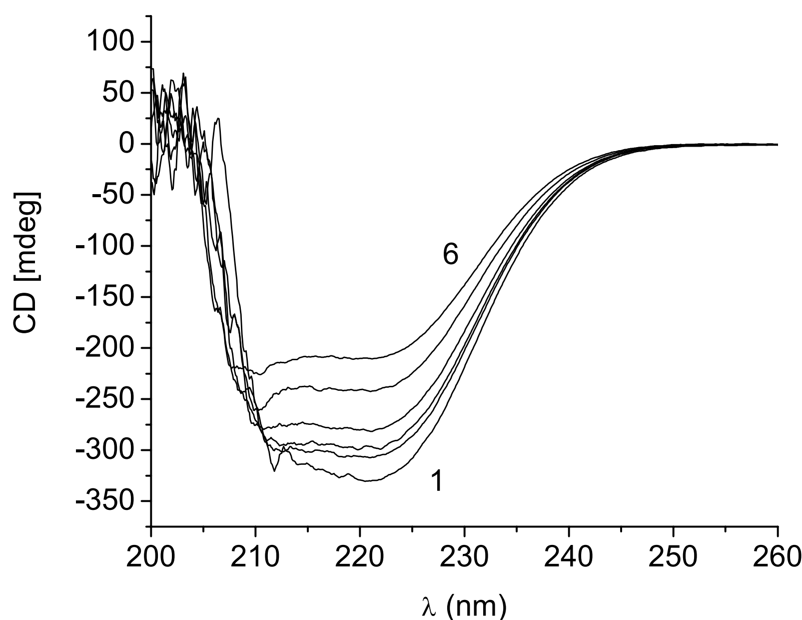



Fig. 8 – CD spectra of the system **I**-BSA at increasing concentration of the ligand. [BSA] = $3*10^{-6}$ M; 1-6 : d/p = 0; 0.46; 0.62; 1.16; 2.33; 2.5; respectively.

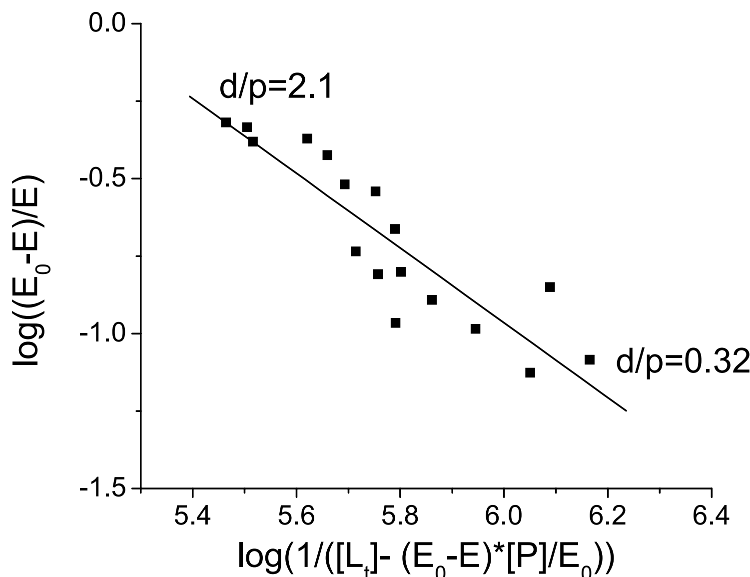



Fig. 9 – Experimental CD data for the system **I**-HSA fitted to eq. (6) adapted to CD data (ellipticities).

The α-helix contents of free and combined BSA/HSA were calculated from mean residue ellipticity values at 222 nm using the following equation: [32]

$$\alpha\text{- helix } \% = \frac{MRE_{222} - 2340}{30300} \times 100 \qquad (11)$$

At the molar ratio of drug/protein d/p= 2.36, the decrease of α-helical content is about 0.8 % for BSA and about 0.29 % for HSA and compound **I**.

The binding of **I** to BSA/HSA caused a decrease in band intensity, without any significant shift of the peaks. The small decrease of the CD signal indicated that after the binding of **I** to the two proteins, the secondary structure of BSA / HSA remains predominantly α-helix.

## EXPERIMENTAL

Bovine serum albumin (BSA, Fraction V, approximately 99%) and human serum albumin (HSA, fatty acid free < 0.05%) were obtained from Sigma Chemical Company, St Louis, USA. Their stock solutions of $3 \cdot 10^{-6}$ M were prepared by dissolving protein in doubly distilled water, stored at 0–4 ºC. The phenoxathiin derivative was synthesized as previously described.[10] The solutions of 2-carboxyphenoxathiin, BSA and HSA were prepared in pH 7.4 phosphate buffer.

Fluorescence measurements were performed on a Jasco FP-6300 spectrofluorimeter at room temperature (25°C) in the range 300–550 nm using the excitation wavelength of 286 nm.

The CD measurements were made on a Jasco J-815 CD spectrometer using a 1.00 cm cell at 0.2 nm intervals, with three scans averaged for each CD spectrum in the range 200–260 nm; the results are expressed as ellipticity ([$\theta$]) in millidegrees.

BSA and HSA concentrations were kept fixed at $3 \cdot 10^{-6}$ M and the drug concentrations were varied to ensure a drug to protein ratio, d/p, in the range 0-3.

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