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Spectroscopic Analysis of the Binding of Amylobarbitone, Secbutobarbitone, Pentobarbitone, Phenobarbitone and Quinalbarbitone to Human Serum Albumin

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Abstract

The binding of five barbiturates: amylobarbitone, secbutobarbitone, pentobarbitone, phenobarbitone and quinalbarbitone to human serum albumin (HSA) was studied by difference spectroscopy and spectrofluorimetric titrations. There were no changes in the HSA spectral properties. Our result suggest that there are two classes of binding sites on HSA for these barbiturates. A detailed investigation of the effect of their binding to HSA by deconvoluted spectra, suggests that the interaction of barbiturate-HSA takes place principally on the subdomain IIIA of HSA.

Barbiturates can produce all degrees of non-specific depression of the central nervous system (CNS), ranging from mild sedation to general anaesthesia (Goodman & Gilman 1990). The degree of depression depends not only on the specific barbiturates, but also, on doses, routes of administration, preconditioned excitability of the CNS at the time of drug administration and existing tolerance induced by previous use. Therefore the barbiturates are often chosen for their specific activity (Buch et al 1970; Breimer 1977).

Tissue distribution of administered drug is often affected by three physical-chemical factors: binding to plasma protein, lipid solubility and degree of ionization (Goldbaum 1954; Waddell & Butler 1957). Barbiturates are known to be bound to plasma albumin to varying extents and are also known to have different lipid solubilities which affects interaction with the hydrophobic regions of the plasma proteins (Bustamante & Selles 1986; Buch et al 1990). The degree of lipophilicity correlates roughly with the extent of binding to plasma protein extracellularly and to cytochrome P450 intracellularly for metabolism (Freudenthal & Carroll 1973).

The interactions of amylobarbitone, pentobarbitone and phenobarbitone with human serum albumin (HSA) (Branstad et al 1972; Nilsson et al 1973), as well as to blood cells and other plasma proteins (Ehrebo & Odar Cederlof 1973) have been studied.

In this paper we report the results of a study of the interactions of five barbiturates with human serum albumin (HSA). The barbiturates studied were: amylobarbitone (5-ethyl-5-isopentyl barbituric acid), secbutobarbitone (5-ethyl-5-secobutyl barbituric acid), pentobarbitone (5-ethyl-5-1′-methylbutyl barbituric acid), phenobarbitone (5-ethyl-5-phenyl barbituric acid) and quinalbarbitone (5-allyl-5-1′-methylbutyl barbituric acid). This study was carried out because the differential degree of CNS depression inducible with various barbiturates depends on the concentration of free drug in plasma, and further their tissue distribution is affected by their degree of binding to plasma protein, mainly to HSA, since this is quantitatively and qualitatively the most important plasma protein (Breimer 1977).

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We used difference spectroscopy and spectrofluorimetric titration, because these techniques are particularly suitable for binding studies if the unbound ligand can be measured (Harris & Bashford 1987). Moreover, to obtain additional information on the changes of UV-vis absorption bands modified in the binding process, spectra were deconvoluted into Gaussian curves following the procedure previously described (Garcia del Vado 1992; Vilanova et al 1992).

Therefore, the aim of the present work was not only to calculate the binding constant of each barbiturate, but also to give an interpretation for the mechanism of the binding process based on the UV-vis and fluorescence spectral changes.

Materials and Methods

Materials

Human serum albumin (HSA) essentially fatty acid-free, Fraction V (Lot. no A-1887), from Sigma Chemical Company (St Louis, MO, USA). Amylobarbitone, secbutobarbitone, pentobarbitone, phenobarbitone and quinalbarbitone were obtained from the Social Institute of the Armed Forces. The compounds used in this study were of the highest commercial purity available and were used as received.

The concentration of HSA was calculated from its UV-vis spectrum using a molar extinction coefficient at 278 nm of 36600 L mol^{-1} cm^{-1} (Peters 1975).

Each stock solution of barbiturate was prepared in phosphate buffer 0·061 M, containing 0·1 M NaCl, pH 7·4 ± 0·1 at 25°C.

All solutions were prepared in phosphate buffer, pH 7·4 ± 0·1 measured using a Crison 2001 pH meter at 25°C.

Absorption spectra were recorded at 25°C with a Uvicon-940 spectrophotometer. The number of bands and their

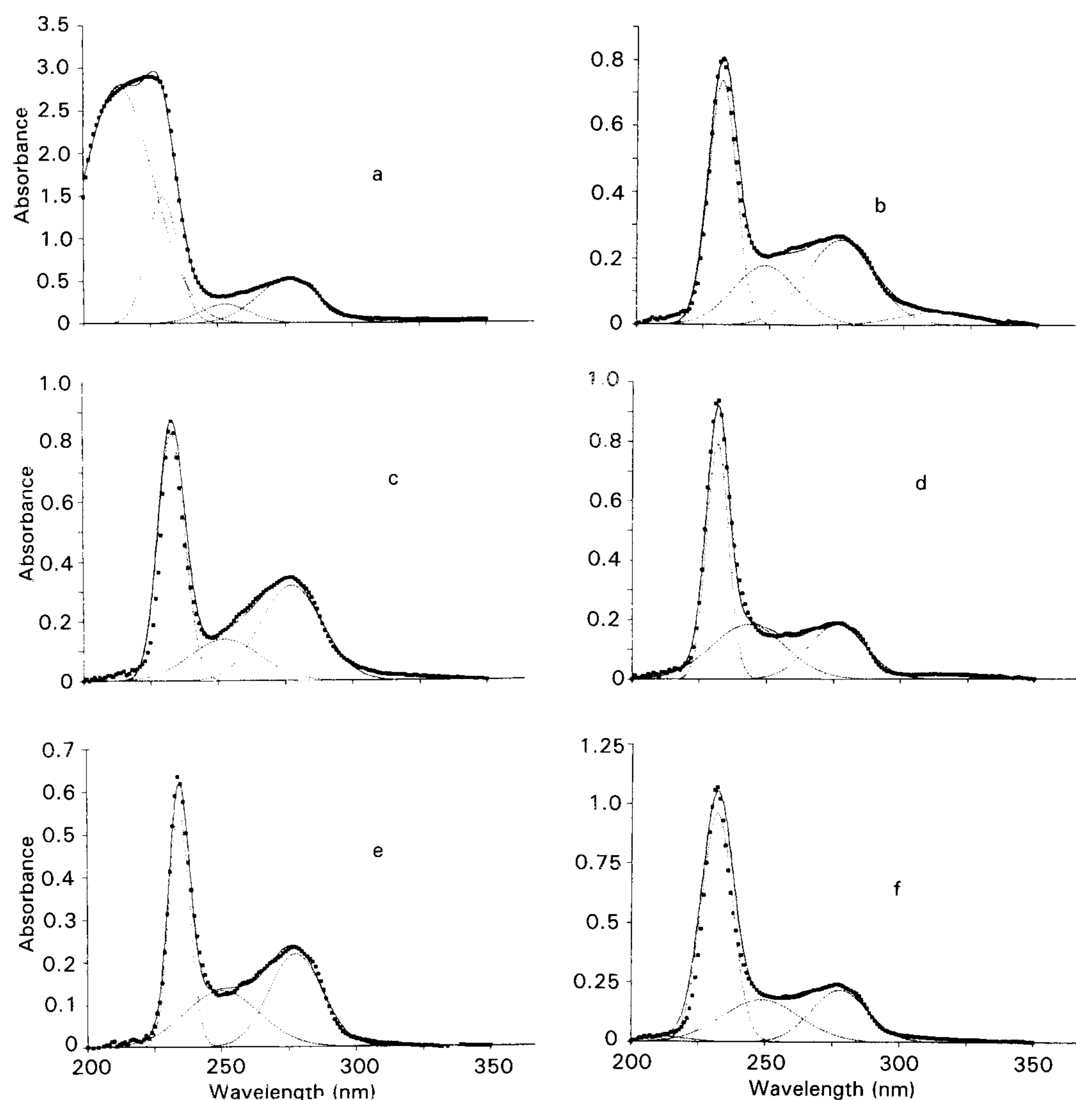

FIG. 1. Deconvoluted spectra of HSA and HSA-barbiturate mixtures. In all experiments HSA protein concentration was kept constant (at 0·01 mM) and the final concentration of barbiturates was approximately 0·2 mM, the concentration in the reference cell at the end of the titration.

position in the spectrum were determined by multiple differentiation. Spectra were deconvoluted into Gaussian curves to examine the modified bands that appear upon adding the drug.

The fluorescence measurements were carried out using a Perkin-Elmer MPF-44A spectrofluorimeter, equipped with a jacketed cell-holder connected to a constant temperature bath thermostated to 25 ± 0·5°C.

An attempt was made to model the reduction in fluorescence intensity observed in the protein by treatment with barbiturates. Fluorescence-quenching experiments were performed using two excitation wavelengths, 277 nm (the wavelength of maximum absorption for HSA) and 290 nm (the maximum absorption for tryptophan). The slit widths were placed at 7 nm for excitation and 6 nm for emission.

Binding measurements

For absorbance measurements, typically 2·0 mL 0·01 mM HSA solution was placed in a 1-cm square quartz sample cell and 2·0 mL buffer was placed in the reference cell. Aliquots of 50 or 100 μL of a solution containing 0·01 mM HSA and 0·5 mM barbiturate were added to the sample cells while the same aliquot containing only 0·5 mM barbiturate was added to the reference cell. After mixing, the absorption spectra were recorded. A similar procedure was followed for the fluorescence measurements as described previously (Gonzàlez-Jimènez et al 1991, 1992).

We calculated the fraction of ligand bound from their respective UV difference spectra as described by Miller et al (1982). The difference of absorbance was plotted as a function of barbiturate concentration at a constant protein concentration. When the molar barbiturate to HSA ratio is small, there was an initial linear change in the difference spectrum. This indicated that under these conditions all of the ligand is stoichiometrically bound to the protein. From this region of the titration an extinction coefficient in terms of the change in absorbance can be obtained for the bound barbiturate (Harris & Bashford 1987). This coefficient was

Table 1. Positions and shape of the absorption bands of all deconvoluted spectra into Gaussian distribution curves. The first figure is the maximum wavelength (nm), with the amplitude and width, respectively, given below.

	I	II	III	IV
HSA	213 2·77; 13·12	229 1·48; 5·70	277 0·51; 11·52	252 0·22; 10·34
Amylobarbitone				
alone	204 2·13; 10·77	237 0·43; 9·27		
with HSA	232 0·73; 5·16	277 0·25; 17·71	248 0·18; 11·84	
Secbutobarbitone				
alone	206 2·06; 12·85	237 0·46; 7·66		
with HSA	233 0·82; 4·82	277 0·32; 12·45	252 0·15; 7·83	
Pentobarbitone				
alone	202 1·72; 14·59	237 0·37; 11·12		
with HSA	232 0·76; 5·40	277 0·21; 12·33	247 0·17; 10·32	
Phenobarbitone				
alone	208 0·41; 9·60	237 1·14; 11·11		
with HSA	234 0·57; 4·12	277 0·23; 11·35	251 0·13; 10·21	
Quinalbarbitone				
alone	202 1·57; 14·72	237 0·44; 8·11		
with HSA	230 0·93; 6·01	277 0·24; 12·13	250 0·21; 10·71	

used to calculate the amount of ligand bound to HSA at later points in the titration, where unbound ligand occurred. Since the total drug is known in each point of the titration, the free drug can be calculated from the difference between the total drug and the bound drug.

Analysis of results

The binding parameters for each individual class of binding sites was calculated according to the Scatchard procedure (Scatchard 1949) using nonlinear least-squares regression, and the BDATA software package (EMF Software 1984).

Each binding experiment was performed at least four times. Results are given as mean ± s.e. and Student's *t*-test was used to compare means.

Results

Deconvolution of the spectra

All spectra were analysed in greater detail by deconvolution. Fig. 1a depicts the deconvoluted spectra of 0·01 mM HSA in phosphate buffer. This spectrum can be deconvoluted into four bands; however, as shown in Table 1, UV-vis spectra of the barbiturates have only two bands.

Fig. 1 b–f, shows the UV-vis spectra at the final point of the titration of HSA with barbiturates. All spectra were deconvoluted into four bands, denoted by I, II, III and IV. Information on these deconvoluted spectra are also included in Table 1.

As can be seen from the spectra, bands I and II are markedly affected by the addition of the barbiturates to HSA. The intensity of both bands decreases dramatically; furthermore the apparent experimental maximum of band II gradually shifted to longer wavelengths, since all barbiturates studied show a maximum at 237–238 nm (Fig. 2). Bands III and IV are only slightly affected by successive additions of barbiturates.

Ultraviolet difference spectra

No changes were noted in the spectral shape of the HSA absorption band (277 nm) in the absorption spectra of aqueous solutions of barbiturates and HSA. A new absorption band becomes apparent when barbiturates are bound to HSA. This band shows a maximum that is gradually shifted from 228 to 232 nm on addition of barbiturates. This effect can also be seen in the deconvoluted spectra of Fig. 1. This effect can be seen in Fig. 3, which is the difference spectrum obtained on titration of HSA with phenobarbitone. The spectrum shows a minimum, which is gradually shifted to higher wavelength on addition of barbiturate and is produced by interaction of HSA with barbiturate.

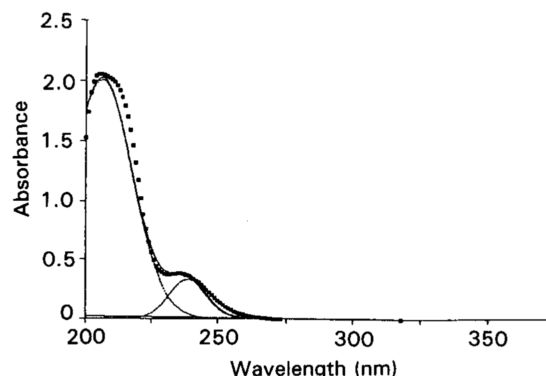

FIG. 2. Deconvoluted spectrum of 0·2 mM amylobarbitone. This concentration is the same as in the reference cell at the end of the titration.

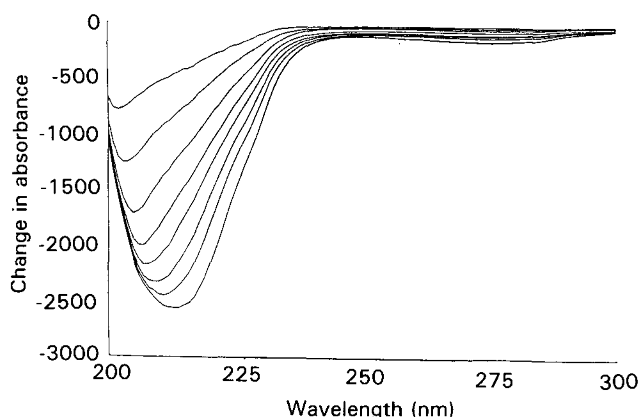

FIG. 3. Difference spectra generated by adding aliquots of 0·1 mL 0·5 mM phenobarbitone to 2 mL 0·01 mM HSA.

Fig. 4 shows the spectrophotometric titration of HSA with barbiturates at 220 nm, which is the wavelength where the overlap between the HSA and barbiturate absorption bands is minimum. The extinction coefficient of each bound drug can be obtained from this figure, as described in the Methods section. The initial part of the curves, at barbiturate concentrations < 0·05 mM, is the same for all, except for secbutobarbitone, which is slightly different. Thus, the respective molar extinction coefficients for the bound drug have the same order (Table 2).

From these values, the saturation function v, the number of moles ligand bound (mol protein)$^{-1}$, can then be obtained (Fig. 5). Such plots exhibit a nonlinear profile, indicating the existence of two classes of binding sites on the protein for the barbiturates studied. The numerical values for the binding parameters are summarized in Table 3.

Fluorescence quenching

We found that over the concentration range studied, the fluorescence intensity of HSA is quenched between 10 and 20%, depending on the barbiturate used. This effect is shown in Fig. 6, where the fluorescence intensity, plotted as a function of the molar ratio of barbiturate to albumin, does not depend on the excitation wavelength. Even with a molar ratio of 3, fluorescence is nearly linearly quenched for both excitation wavelengths. The fluorescence emission spectra of HSA were also evaluated at the same emission wavelengths; no significant changes in the shape or the emission wavelength of these spectra were observed.

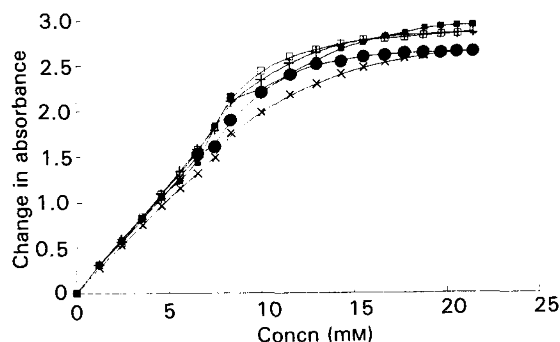

FIG. 4. Spectrophotometric titration of HSA with amylobarbitone (■), secbutobarbitone (+), phenobarbitone (●), pentobarbitone (□) and quinalbarbitone (×) at a constant protein concentration (0·01 mM) and at room temperature.

Table 2. Molar extinction coefficients for the bound barbiturates, obtained from Fig. 4.

	Molar extinction coefficient (10^{-3} × L mol^{-1} cm^{-1})
Amylobarbitone	22·63 ± 1·48
Secbutobarbitone	24·06 ± 0·42
Pentobarbitone	21·35 ± 1·49
Phenobarbitone	23·38 ± 2·30
Quinalbarbitone	23·07 ± 0·66

Each value is the mean of four determinations ± s.d.

Discussion

The binding of barbiturates to HSA can be studied from spectroscopic measurements, since changes in the spectral properties of HSA have been detected when barbiturates are present. There is a trend in the relative intensity of bands I and II. Band I decreased as the concentration of these ligands increased, whereas band II located at 228 nm also undergoes a 6–8 nm red-shift upon addition of barbiturate. This is a consequence of the unbound ligand, since barbiturates show a typical band located at 237 nm. Thus, this region of the UV spectrum was used to estimate the binding parameters.

Thus, from the spectral changes expressed in the form of a titration curve, it seems that at low concentrations there are no significant differences in the affinity of these barbiturates for HSA, as the corresponding molar extinction coefficients for the bound barbiturate are of the same order of magnitude. Hence, for this concentration range, the binding of these compounds to HSA is practically independent of the compound size and the electronic nature of their substituents.

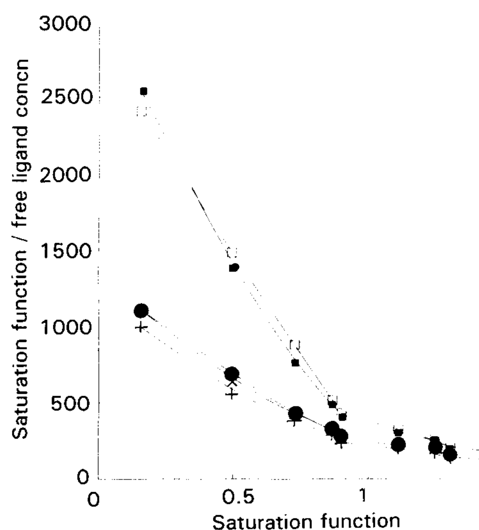

FIG. 5. Scatchard plots for the binding of amylobarbitone (■), secbutobarbitone (+), phenobarbitone (●), pentobarbitone (□) and quinalbarbitone (×) to HSA.

Table 3. Association constants for the binding of barbiturates to HSA.

Barbiturate	Association constant	
	High-affinity ($10^5 \times M^{-1}$)	Low-affinity ($10^4 \times M^{-1}$)
Amylobarbitone	12·68 ± 1·19	3·33 ± 0·23
Secbutobarbitone	7·81 ± 0·51	3·95 ± 0·06
Pentobarbitone	10·90 ± 2·01	2·70 ± 0·35
Phenobarbitone	9·81 ± 1·91	3·41 ± 0·70
Quinalbarbitone	16·81 ± 1·72	2·60 ± 0·11

Each value is the average of four determinations ± s.d.

Moreover, examination of the drug-induced changes at the top of the titration curve indicates that in the barbiturates studied, some differences can be detected in the maximum absorption differences as the concentration increased. Such differences can also be perceived from the values of the primary binding constants. The maximum difference in absorption is obtained when no change is observed on addition of ligand, that is, when the protein has been saturated.

The decreasing order of the binding constants to the high affinity sites estimated from their respective molar extinction coefficients of the bound ligand is: amylobarbitone > quinalbarbitone > pentobarbitone = phenobarbitone > secbutobarbitone. This order is also in agreement with the results of Branstad et al (1972).

Regardless of the compounds studied here, the shape of the Scatchard plots suggests two classes of binding sites, compartments of apparent high and low affinity as had also been found previously for amylobarbitone, pentobarbitone and phenobarbitone binding to HSA in the pH range 7·33–8·38 (Branstad et al 1972; Nison et al 1975). However, several discrepances have been found for the binding parameters, possibly due to the interaction of the protein molecules with the fatty acids, since the albumin used in this work is essentially fatty acid-free, and it is known that there is a high affinity of fatty acids for HSA (Hamilton 1992).

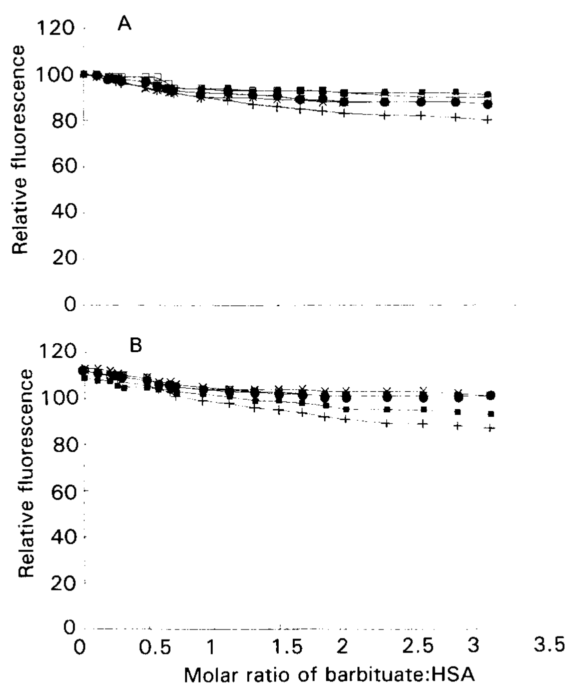

FIG. 6. Fluorescence titration of HSA with amylobarbitone (■), secbutobarbitone (+), phenobarbitone (●), pentobarbitone (□) and quinalbarbitone (×) at 25°C and with excitation wavelengths of 277 (A) and 290 nm (B). In all cases the emission wavelength was 335 nm.

The magnitude of these constants reflects the dependence of binding on hydrophobicity. Of the five barbiturates tested, all have an ethyl group at position five, except quinalbarbitone, which has an allyl group at this position. This effect is probably due to the greater hydrophobicity of the unsaturated substituents.

In addition, the number of carbon atoms and the position of the methyl group in the branched chain are also important, since hydrophobic interactions make a major contribution to reversible binding between ligands and albumin. Thus, in agreement with the results of Hasegawa et al (1991) and Panjehshahin et al (1991), those barbiturates with a low lipid : water partition coefficient are bound to a lesser extent than those with a high lipid : water partition coefficient.

Recently, the three-dimensional structure of HSA has been determined crystallographically (He & Carter 1992). The structure comprises three homologous domains, denoted as I, II, and III, and each domain is a product of two subdomains, A and B. Based on preliminary binding studies, the same authors showed that the principal binding regions on HSA were located in subdomains IIA and IIIA.

Fluorescence quenching experiments were performed to gain information on the microenvironment of the chromophore groups on HSA. The environment of the tryptophan residue has a direct influence on the wavelength of maximum fluorescence emission, with a shift to longer wavelengths as the tryptophan residue becomes more exposed to the solvent (Eftink & Ghiron 1977; Clark et al 1987; Higashijima & Ferguson 1991).

According to the above considerations and based on the spectrophotometric and spectrofluorimetric studies, it seems reasonable to propose that the binding of barbiturates to HSA could take place principally on the subdomain IIIA. Firstly, the absence of changes in the emission maxima after each titration suggests that the tryptophan's microenviroment did not suffer any significant changes, since the tryptophan residue located at position 214 in HSA plays an important structural role in the formation of the subdomain IIA binding site (He & Carter 1992). Moreover, no fluorescence quenching was observed until the ligand/protein ratio is approximately 0·3, and as the protein concentration is constant, such ligand concentration does not correspond with the values of the high affinity binding sites. Secondly, as observed by fluorescence, the biggest differences in the deconvoluted UV-vis spectra were also observed in the intensity of the bands I and II, which is in the region of the spectrum where the barbiturate absorbance is most intense. Based on similar UV-vis and fluorescence spectral changes, this subdomain or a site close to it was proposed for binding of chlorpheniramine to HSA (Gonzàlez Jimènez 1994). Furthermore, Hamilton (1992) using NMR, has also identified multiple high-affinity

binding sites on the subdomain III of bovine serum albumin for fatty acids.

In conclusion, the results of the present study as obtained by UV-vis spectrophotometric titrations and fluorescence quenching measurements, provide evidence for the interaction of the barbiturates mainly with subdomain IIIA on HSA, because of their ability to bind several moles of the same ligand, with high affinity.

Acknowledgement
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