Biophysical Chemistry 10 (1979) 389–396

THE INTERACTION OF SERUM ALBUMINS WITH VARIOUS DRUGS IN AQUEOUS SOLUTION. GEL PERMEATION, CALORIMETRIC, AND FLUORESCENCE DATA

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Received 7 September 1978

Thermodynamic data relative to the reversible interaction between human or bovine serum albumin and some organic ligands (S- and R-warfarin, d- and l-oxazepam hemisuccinate, phenyl-butazone, fluorescein) in dilute aqueous solution were determined by means of gel permeation chromatography and microcalorimetric measurements. From an analysis of these data and on the basis of fluorescence titrations the identity of the "primary" binding site on the proteins for some ligands was evidenced, while in other cases a cooperative binding of two different ligands to different binding sites could be discerned.

1. Introduction

A unique feature of mammalian plasma albumins is their capacity to strongly bind a variety of organic ligands. Studies carried out using human (HSA) and bovine (BSA) serum albumins indicate that these macromolecules contain a number of binding sites of varying affinity, depending of course on the ligand.

To account for the versatility of binding exhibited by albumins, it has been proposed that these carrier-proteins have a rather flexible structure and easily adapt their conformation, at least locally, to optimize interaction with a given ligand molecule. Most of the ligands of pharmacologic interest are of predominantly apolar, aromatic nature so that their binding onto the protein sites would be mainly driven by van der Waals and hydrophobic forces. It is then of interest to inquire if on HSA and BSA ligands which are structurally not too dissimilar, utilise identical or different primary-binding sites. If the sites are identical then ligands should compete with one another. If the sites are different, one may nevertheless expect a cooperativity to exist between sites in view of the alleged conformational ductility of serum albumins. Along these lines, a comparative analysis has been carried out in our laboratories on some physico-chemical aspects of the interactions in dilute aqueous solution of HSA and BSA, respectively, with the ligands: S- and R-warfarin, d- and l-oxazepam hemisuccinate, phenylbutazone and fluorescein.

The compounds considered, although of quite different pharmacological activity, have some structural resemblance with each other and all are negatively charged ar around pH 7.

Moreover, warfarin and oxazepam hemisuccinate can be used in their pure d- and l-forms, so that possible stereo-specific interactions with the protein sites could be evidenced.

We wish to report here our results obtained by means of gel-permeation chromatography, calorimetry and fluorescence analysis of the interacting systems mentioned above. Each of the ligands considered binds to more than one site on serum albumins, but generally the affinity to one site prevails on the others. Attention is here confined to HSA and BSA primary binding sites.

Binding studies for each of the ligands mentioned above and HSA or BSA have also been made by different workers [1–11]. To our knowledge, however, a thermodynamic characterization of the interactions

and a deeper insight into the possible interdependence of binding sites on each albumin molecule has as not yet been achieved by others for all drugs considered in this work.

2. Experimental

2.1. Samples

HSA and BSA used in this work were purchased from SIGMA Chem. Co. as crystallized and liophylized samples (cat. n. A-9511 and A-4378 respectively). Generally these samples were used without further purification. Albumin concentration was determined by UV spectrophotometry assuming $E^{1\%}_{1\,cm} = 5.3$ and $E^{1\%}_{1\,cm} = 6.6$ at 279 nm for HSA and BSA respectively. which would correspond to about $\epsilon_{279} = 3.6 \times 10^4$ and $\epsilon_{279} = 4.4 \times 10^4$ for the two albumins. S-warfarin and R-warfarin, as sodium salts, were a kind gift of Endo Lab. Inc.; elemental analysis gave the following results: C = 65.2%, H = 4.72%, for S-warfarin and C = 65.1%, H = 4.78% for R-warfarin, in good agreement with the calculated values (C = 65.5%, H = 4.90% for the hydrated salt: $C_{19}H_{15}O_4Na \cdot H_2O$). According to these data the extinction coefficient at 308 nm (pH = 7.4) resulted to be 13 900 M^{-1} cm^{-1} in agreement with literature data [2].

d-Oxazepam and l-oxazepam hemisuccinic acids were a kind gift of Ravizza S.p.A.; they were solubilized as potassium salts in 0.065 M phsophate buffer which was used as solvent throughout the work; concentration was determined spectro-photometrically using $\epsilon_{231\,nm} = 3.8 \times 10^4$ and $\epsilon_{315\,nm} = 2650$ M^{-1} cm^{-1} values which were obtained from solutions, prepared by weighing, of the dried racemic sample whose purity grade was better than 99%.

Phenylbutazone was a sample purchased from Hoechst in the acidic form; it was solubilized with equimolar NaOH in phosphate buffer; elemental analysis for the salt: C = 68.6% (68.8), H = 6.02% (6.08), N = 8.48% (8.35), the values in parenthesis being calculated ones for $C_{19}H_{20}N_2O_2Na$; concentration was determined spectrophotometrically using $\epsilon_{265\,nm} = 2.0 \times 10^{-4}$ M^{-1} cm^{-1}.

Fluorescein was purchased from Merck as sodium salt and used without further purification; the concentration was determined by UV spectrophotometry using $\epsilon_{490\,nm} = 8.4 \times 10^4$ M^{-1} cm^{-1}.

2.2. Gel permeation chromatography measurements

The affinity of the drugs considered for HSA and BSA was determined by the gel permeation chromatography technique described by Hümmel and Dreyer [12]. A column of about 0.6 cm inner diameter, 40 cm height, filled with Sephadex G-25, was used at room temperature (23 ± 2°C). All data were plotted according to Scatchard. The experimental data were matched theoretically assuming in each case an integer number of non-interacting binding sites and the appropriate binding constants. In some cases (R-, S-warfarin, phenyl-butazone and d-oxazepam hemisuccinate with both albumins) the presence of a "primary" site with higher binding constant was evidenced and the relative ΔG values are reported in table 1; in other cases (l-oxazepam hemisuccinate and fluoresceine with both albumins) the linearity of the plots suggested the presence of more than one site of comparable affinity and the corresponding mean ΔG values are reported in table 1. Obviously the assumption of mutual independence of the binding sites is somewhat arbitrary but, (as no case of relevant positive cooperativity emerged from those data), the uncertainty on the binding constant relative to the primary site cannot be so high to strongly affect the corresponding ΔG value.

For what concerns the affinity of R- and S-warfarin for HSA our data do not show significant differences between the two isomers, within the experimental error, and agree with the data of O'Reilly [3,4]. The few data obtained by dialysis for the warfarin-BSA systems were less accurate but the affinity for the protein appeared very close to that for HSA.

On the contrary, a significant difference between the two isomers was evidenced in the case of d- and l-oxazepam hemisuccinate with regards to their affinity for both HSA and BSA, as already reported [8,9] (even if our data for the d-isomer do not agree quantitatively with those of the literature).

In the case of fluorescein the results show a substantial identity in the affinity for the two albumins, in agreement with literature data [4] if account is made for differences in working temperatures.

The Scatchard plots for d- and l-oxazepam hemisuccinate and phenylbutazone with both albumins are reported as examples in figs. 1 and 2.

Table 1

drug	HSA			BSA		
	$-\Delta G$ kcal/mol	$-\Delta H$ [a] kcal/mol	ΔS e.u.	$-\Delta G$ kcal/mol	$-\Delta H$ kcal/mol	ΔS e.u.
d-oxazepam hemisuccinate	6.5	0	22	6.5	6	2
l-oxazepam hemisuccinate	5	2	10	5.5	3	8
R-warfarin	7.5	3	15	7.5	5	8
S-warfarin	7.5	5	8	7.5	5	8
phenylbutazone	7.5	1	22	8.5	8.5	0
fluoresceine	6.5	4	88	6.5	3.5	10

a) These values are normalized permole of protein, and, since they are taken at $r = 1$, per mole of bound ligand too.

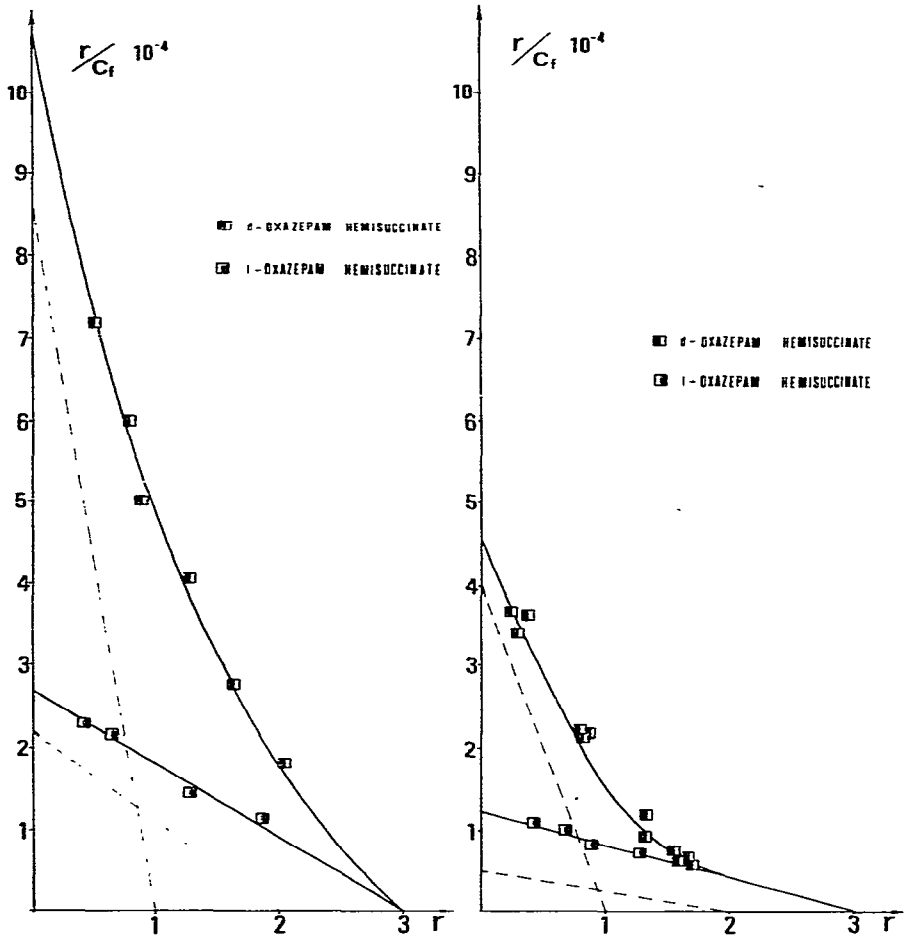

Fig. 1. Scatchard plots frc .n GPC data relative to the oxazepam hemisuccinate-BSA (left) and HSA (right) systems. Full lines are calculated in the hypothesis of independent sites (as shown for the d-antipode by dashed lines).

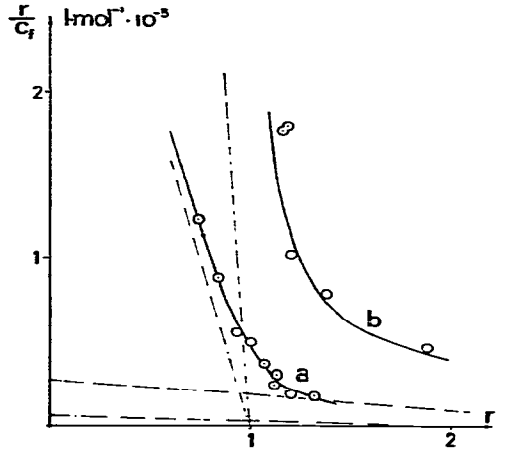

Fig. 2. Scatchard plots from GPC data relative to the interaction of phenylbutazone with HSA (a) and BSA (b). Full lines calculated as described in the case of fig. 1.

2.3. Microcalorimetric measurements

These measurements were performed using a LKB 10700 flow microcalorimeter, except in the case of the systems warfarin-albumin, for which a LKB 10700 batch microcalorimeter was used.

The experimental heats of mixing the protein and drug solutions were normalized per mole of protein.

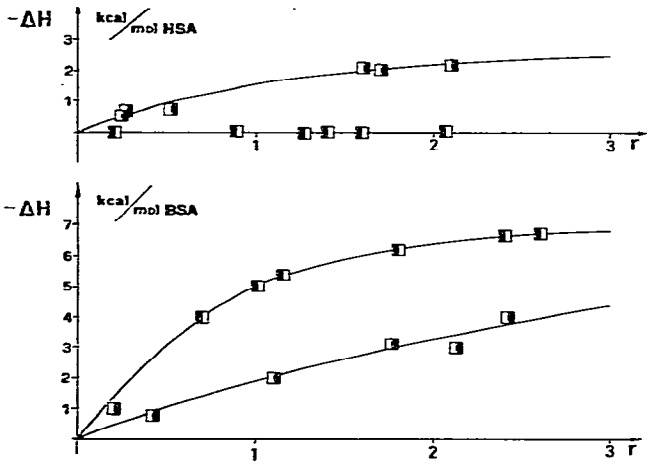

Fig. 3. Calorimetric data relative to the interaction of d- and l-oxazepam hemisuccinate with HSA and BSA. See sect. 2.3.

The heats of dilution of the various drugs were negligible while the calorimetric data were intrinsically corrected for the heat of dilution of the protein. The mean ΔH value for measurements at $r \approx 1$ was taken as the enthalpy of binding relative to the primary site and is reported in table 1. Figs. 3 and 4 show, as examples, the ΔH data for d- and l-oxazepam hemisuccinate and phenylbutazone, as experimental heats normalized per mole of protein, plotted in function of r, calculated according to GPD data (r is the ration between bound ligand concentration and protein concentration).

2.4. Spectrophotometric and spectrofluorimetric measurements

The spectrophotometric measurements were performed by means of a Coleman-Hitachi EPS-3T spectrophotometer using rectangular quartz cuvettes of 1, 2, 5 and 10 mm path length. This technique was used for monitoring the fluorescein-albumin complexes (see results and discussion), as well as for routine concentration determinations.

The spectrofluorimetric measurements were performed by means of a Perkin Elmer MPF-3L spectrofluorimeter using quartz cuvettes (1 cm × 1 cm section). The experimental fluorescence intensities F were corrected for the inner filter effect:

$$F \propto I_0 \, \phi \, C_F \times 10^{-A/2}$$

so that

$$\phi \propto (F/I_0 C_F) \times 10^{A/2}$$

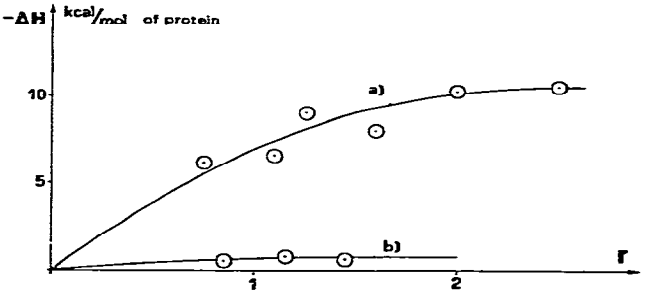

Fig. 4. Calorimetric data relative to the interaction of phenylbutazone with BSA (a) and HSA (b). See sect. 2.3.

with I_0 the exciting light intensity, C_F the molar concentration of fluorescein or other drug (bound and free), A the sum of optical densities of the species in solution at the excitation and emission wavelengths (the last addend being usually zero), and ϕ the mean quantum yield of the free and bound species of fluorescein or other fluorescent drug in solution. The results are reported in figs. 5, 6 and 7.

3. Results and discussion

3.1. GPC and calorimetric data

The results of GPC measurements, from which the binding constants and hence the associated ΔG values have been evaluated, and of the microcalorimetric measurements, all collected at 25° and relative to the first, strongest binding site (see sect. 2) in each case, are reported in table 1.

A few interesting features appear evident from these results.

a) The affinity varies going from one ligand to another (not markedly though) but for each given ligand the results are nearly the same, at least within experimental errors, for HSA and BSA.

In this connection it is relevant to point out that binding by HSA or BSA of the drugs considered is certainly not a simple "polyelectrolytic" effect. For instance, warfarin does not interact detectably with poly-L-lysine or is only weakly bound by poly(vinylbenzyltriethylammonium bromide), a strong cationic polyelectrolyte, under similar experimental conditions.

b) In the case of d-oxazepam hemisuccinate and of phenylbutazone the ΔG of binding onto HSA and BSA are built up by quite different ΔH and ΔS contributions, respectively.

c) For what concerns the identity (energetically speaking at least) of the "primary" sites, data of table 1 suggest the following preliminary conclusions:

1) d-oxazepam hemisuccinate would bind to different primary sites on HSA and on BSA, respectively.

2) phenylbutazone would also bind to different primary sites on HSA dn on BSA, respectively.

3) S-warfarin would bind to homologous primary sites on HSA and BSA. The same would apply in the case of fluorescein, always on the basis of GPC and calorimetric data.

The situation with l-oxazepam and with R-warfarin is less clear cut on the basis of the data of table 1. It appears probable, however, that each of these two ligands has the same "primary" site on both proteins.

The question remains, of course, about which sites (if any) are just the same for the different ligands on each albumin molecule, although some exclusion-criteria might be already deduced from points 1)-3) above (see also table 1). In this context interesting information may be derived by fluorescence intensity measurements carried out using two ligands at the same time.

3.2. Fluorescence data

In fig. 5 the intensity of fluorescence of S-warfarin in the presence of HSA and BSA, respectively, is given as a function of added oxazepam hemisuccinate concentration. These data indicate that the latter drug would "displace" S-warfarin from both proteins, the d-form being the more efficient one in this respect particularly with HSA.

Recalling remarks 1) and 3) given in sect. 3.1 above, one should conclude however that *either* with HSA *or* with BSA such a "displacement" does not result by a direct competition between the two drugs but probably via a negative cooperativity effect.

Wishing to shed more light on this interesting aspect, we have used another fluorescent probe, fluorescein. This dye when bound to its primary-sites on HSA and BSA (which would be "homologous" sites, according to our GPC, calorimetric and fluorescence data) has a quantum yield less than half that of the free form in water. The results are given in figs. 6 and 7. From fig. 6 it appears that S-, and R-warfarin as well as phenylbutazone directly compete with bound fluorescein for the same primary site on HSA. On the contrary, upon addition of l-oxazepam hemisuccinate there is a *decrease* in the fluorescence intensity of fluorescein. Assuming that the more extensive and/or the tighter is the binding of fluorescein by HSA the lower is its relative fluorescence intensity, then one may think that fluorescein and l-oxazepam hemisuccinate are bound onto two different primary sites, say A and B, which are interdependent in the sense that occupancy of B by the latter drug positively influences the affinity of A for fluorescein.

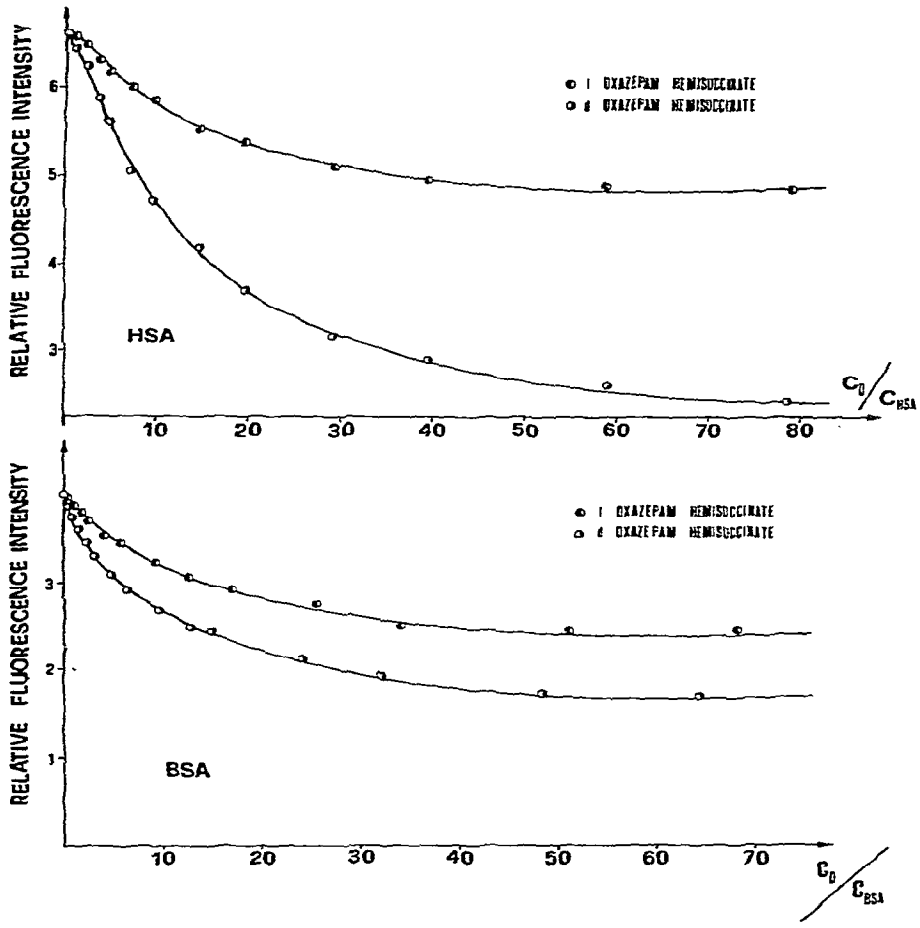

Fig. 5. Relative fluorescence intensity of S-warfarin in the presence of serum albumins as a function of the ratio between concentration of d- and l-oxazepam hemisuccinate added and protein concentration. The fluorescence intensity of a solution of S-warfarin in absence of the protein at fixed concentration is taken as unitary value. Concentration of S-warfarin 0.27×10^{-5} M, and 0.75×10^{-5} M, respectively, concentration of HSA 0.9×10^{-5} M and of BSA 0.75×10^{-5} M. $\lambda_{exc} = 320$ nm, $\lambda_{em} = 385$ nm.

With d-oxazepam hemisuccinate and HSA the situation is more complicate as this drug seems initially to displace fluorescein but afterwards to promote a decrease in its fluorescence (see fig. 6). In any case these results seem to rule out the possibility that the two optical isomers have the same strong binding site on HSA, in agreement with indications from calofimetric data (see table 1). As a consequence, the primary site of l-oxazepam hemisuccinate on HSA is also different from that of warfarin or phenylbutazone (see fig. 6).

In the case of BSA (fig. 7) one may conclude that binding of d- and l-oxazepam hemisuccinate as well as of phenylbutazone induce a favourable effect on the binding site of fluorescein. Comparison of figs. 6 and 7 also confirms that the primary sites for d-oxazepam hemisuccinate on the two albumin molecules must be quite different, in agreement with calorimetric evidences (table 1).

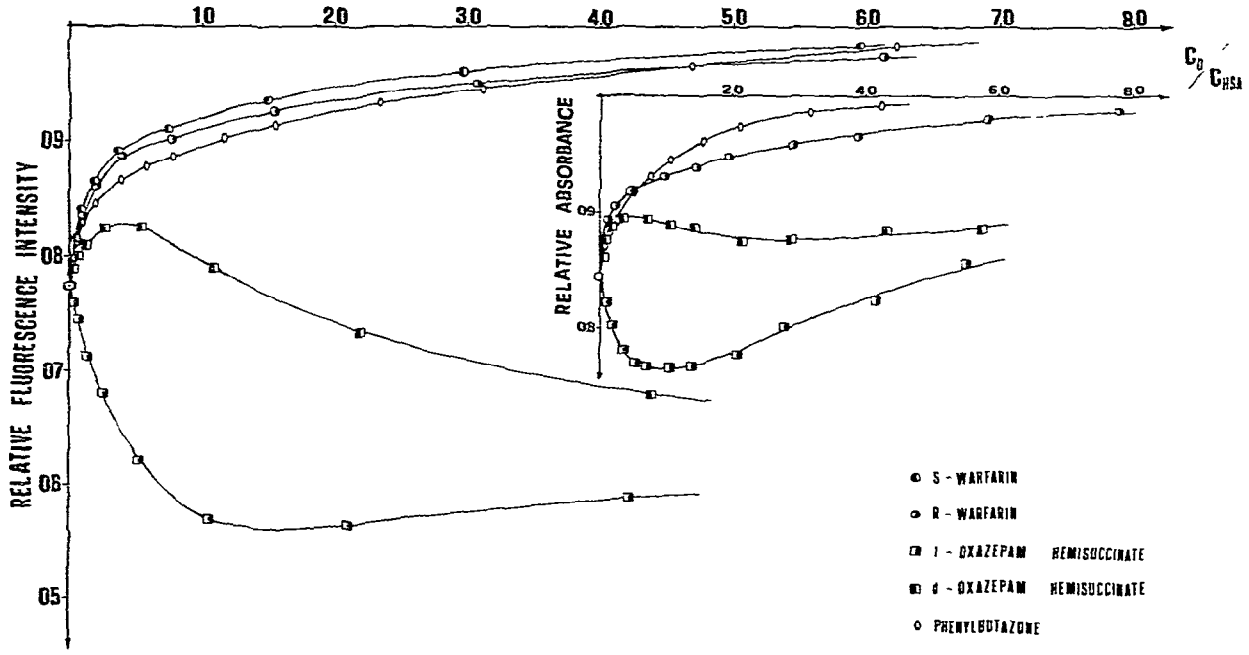

Fig. 6. Relative fluorescence intensity of fluorescein (0.35×10^{-5} M) in the presence of HSA (1.4×10^{-5} M) in function of S- and R-warfarin, d- and l-oxazepam hemisuccinate or phenylbutazone added. Unitary relative fluorescence intensity is that of a solution of fluorescein (0.35×10^{-5} M) in absence of protein; $\lambda_{exc} = 490$ nm, $\lambda_{em} = 515$ nm. Insert: relative absorbance of fluorescein (0.7×10^{-5} M) in the presence of HSA 2.1×10^{-5} M at 490 nm, in function of the same ligands added.

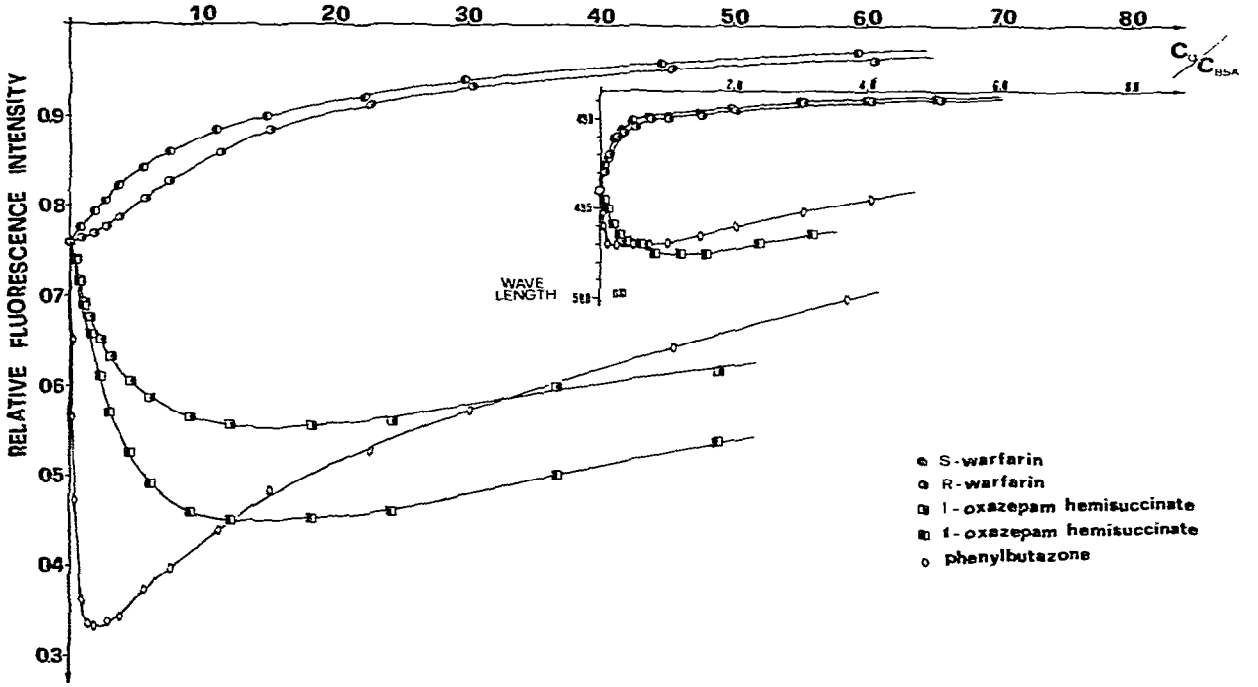

Fig. 7. Relative fluorescence intensity of fluorescein (0.35×10^{-5} M) in the presence of BSA (2.1×10^{-5} M) in function of the ligands added; $\lambda_{exc} = 490$ nm, $\lambda_{em} = 515$ nm. Insert: the maximum wave length for adsorption spectra of fluorescein (0.7×10^{-5} M) in the presence of BSA in function of the ligands added.

Acknowledgement

This work has been sponsored by the Italian Consiglio Nazionale delle Ricerche, Rome.

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