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# Human Serum Albumin Conformational Changes as Induced by Tenoxicam and Modified by Simultaneous Diazepam Binding

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**Abstract**—The binding of tenoxicam to human serum albumin has been shown by affinity chromatography proton titration and equilibrium dialysis to be dependent on the neutral to basic conformational change of the protein. The influence of diazepam on the interaction was also investigated using the same techniques, suggesting that diazepam increases the association of tenoxicam to albumin. Affinity chromatography revealed that the reciprocal effect also occurs. Displacement studies indicated that diazepam causes a significant increase in the affinity of tenoxicam to its main binding site, albumin site I, which is different from the diazepam site (site II). Tenoxicam seemed to cause an allosteric change in the conformation of the protein during its own binding, as did warfarin. The mechanism of this effect was a pH-dependent conformational change of albumin induced by electrostatic forces within the protein. Diazepam induced a distant accommodation of the protein, an effect accompanied by an enhanced inhibition of the release of protons from albumin.

Tenoxicam, an acidic drug of $pK_a = 5{\cdot}3$, is almost completely ionized at physiological pH. It belongs to the chemical class of oxicams and is used for its anti-inflammatory effects (Visher 1987; Barclay & Trabally 1987). Like most non-steroidal anti-inflammatory drugs, tenoxicam binds with high affinity to human serum but it does so with two characteristics (Brée et al 1989). Firstly, it binds not only to site I (the warfarin site) but also to a lesser extent to site II (the benzodiazepine site), although binding to these two sites was initially considered to be exclusive (Sudlow et al 1975, 1976). Secondly, when a low concentration of tenoxicam is used (which implies binding to site I only), this binding is enhanced by diazepam, i.e. by a drug bound only to site II. This strongly suggests that an allosteric effect is reduced from site II to site I.

Allosteric effects are known to occur in some interactions between human serum albumin (HSA) and its ligands (Birkett et al 1977; Sjöholm et al 1979; Wanwimolruk et al 1983; Fitos et al 1986) and are triggered by binding forces such as hydrogen bonds, Van der Waals and electrostatic interactions, and hydrophobic bonds (Kragh-Hansen 1988). The goal of this work was to examine the occurrence of such an allosteric effect and to search for its mechanism. At physiological pH, the problem was complicated by the existence of two conformational states in HSA, namely a neutral (N) and a basic (B) form (Wilting et al 1979, 1980). The effects of diazepam on tenoxicam binding were therefore studied at pH 6·8 (N state) and 9·2 (B state) (Janssen et al 1981; Wanwimolruk & Birkett 1982). Tenoxicam binding was assessed by affinity chromatography, proton titration and equilibrium dialysis.

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## Materials and Methods

### *Chemicals*

HSA (Sigma A-1887 fatty acid-free) was used in equilibrium dialysis and proton titration measurements. HSA used for affinity chromatography studies was from Miles (Elkhart, USA). [$^{14}$C]Tenoxicam, tenoxicam and diazepam were from Hoffmann-La Roche (Basel, Switzerland). [$^{14}$C]Diazepam was from Amersham (UK), warfarin was kindly supplied by Merrell-Toraude (Neuilly-sur-Seine, France). BrCN-Activated Sepharose 4B and Amberlite IRA 400 and IR 120 were from Pharmacia Fine Chemicals (Uppsala, Sweden) and Fluka (Mulhouse, France), respectively.

### *Affinity chromatography*

Binding studies using affinity chromatography were performed according to Lagercrantz et al (1979). Fatty acid-free HSA (1%) was immobilized on BrCN-activated Sepharose 4B. Chromatography was performed at room temperature (21°C) with a Ringer buffer (pH 7·4) containing 0·01% sodium azide. Elution volumes were measured by UV detection (370 nm) or by radioactive liquid scintillation counting.

### *Proton-titration*

An approximately 6% solution of HSA was deionized by passage through a mixed-bed ion exchange column (Amberlite IRA 400 and IR 120, mixed in a 2:1 ratio), until the solution reached a constant conductance. The average pH of the resulting solution was 5·70 at room temperature; it was adjusted to an ionic strength of 0·15 with KCl and a measured HSA concentration of 915 μM (Perkin-Elmer 557 spectrophotometer, $\varepsilon = 35\,400$ at 279 nm). Samples were stored at −20°C until use. Samples of 2 mL deionized and diluted (450 μm) HSA solution and concentrated solutions of tenoxicam (2250 μM), diazepam (750 μM), or warfarin (750

μM) in 0·15 M KCl were mixed in a titration vessel (thermostated at 37°C under nitrogen flow) to a final volume of 6 mL and a final HSA concentration of about 150 μM. The pH of the final solutions was measured using a titroprocessor (Metrohm 670, Herisau, Switzerland). Incremental volumes of 10 μL 0·1 M NaOH (prepared in degassed water) were added, up to a pH value of 10, so covering the pH ranges of the N and B states of HSA. The pH and volume incremental values were then transferred directly to an IBM-AT computer and the value of $Z_H$ was calculated for each pH value (see below).

*Equilibrium dialysis*

Solutions of HSA, tenoxicam and diazepam were prepared in a phosphate buffer at pH 6·8 or 7·4 or in a borate buffer for pH 9·2. Dialysis was performed with a Dianorm (Science Tec, Les Ulis, France) apparatus at 25 and 15°C as previously described (Brée et al 1989). Equilibrium was achieved within 5 h or overnight at 15°C, without apparent accumulation of fluid in the protein side of the dialysis chamber. No significant binding to the dialysis membrane or the cell walls of the apparatus was observed. At the end of experiments the concentration in each dialysis chamber was measured with a liquid scintillation counter (Packard Tricarb 460 CD, Rungis, France).

*Analysis of data*

*Dialysis experiments.* The equilibrium binding data were fitted to the Scatchard model. For m classes of independent binding sites, the bound concentration of drug (B) is related to the free concentration of drug (F) by the relationship:

$$\frac{B}{R} = \sum_{i=1}^{m} n_i \frac{K_i \cdot F}{1 + K_i \cdot F} \quad (1)$$

where R is the concentration of total protein, $n_i$ is the number of binding sites and $K_i$ the association constant of the ith class of sites. To be theoretically valid, the Scatchard model should have integer values for $n_i$, implying the existence of several discrete binding sites. In this case we used $n_i = 1$.

*Proton titration experiments.* The decrease in the number of protons bound to albumin is symbolized by the $Z_H$ parameter (Janssen & Van Wilgenburg 1978). For the protein alone, and assuming that the ligand itself does not bind protons in the pH range investigated, $Z_H$ can be calculated according to the equation:

$$Z_H = -r_{NaOH} \quad (2)$$

where $r_{NaOH}$ is the number of moles of NaOH relative to the number of moles of albumin. When the bound ligand is able to bind protons, the following equation should be used to calculate $Z_H$:

$$Z_H = -r_{NaOH} - (1-\alpha)r_D \quad (3)$$

where α is the degree of dissociation of the ligand and $r_D$ is the number of moles of ligand relative to the number of moles of albumin.

## Results and Discussion

*Affinity chromatography measurements*

The results of tenoxicam binding to the HSA column alone and in the presence of diazepam, as well as the reciprocal experiment, are presented in Table 1 and Fig. 1. Previously, affinity chromatography has allowed, for example, the stereoselective binding of benzodiazepines to be characterized (Fitos et al 1986). Further, warfarin and benzodiazepines were found to increase each other's binding. Here too, tenoxicam (predominantly a site I ligand) and diazepam (a site II ligand) are clearly seen to increase each other's binding to HSA. This affords primary evidence that some allosteric effect may be involved.

Table 1. Binding of tenoxicam and diazepam to HSA and their mutual interaction by affinity chromatography.

| Compound eluted | Elution volume (mL) of eluted compound | | |
|---|---|---|---|
| | Ringer buffer | $10^{-4}$ M diazepam | $10^{-4}$ M tenoxicam |
| Tenoxicam[a] | 43 | 50 | — |
| Diazepam[b] | 100 | — | 128 |

[a] Column 12 × 40 mm, void volume 3 mL; [b] Column 12 × 85 mm, void volume 6·5 mL. Experiments were carried out at room temperature and pH 7·4.

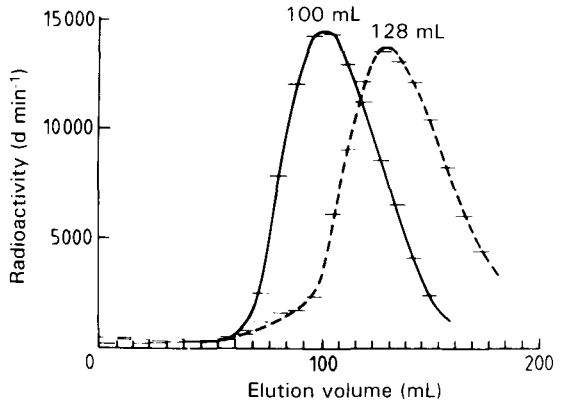


FIG. 1. Radiochromatogram of [$^{14}$C]diazepam on an HSA-Sepharose column. The void volume was 6·5 mL and 7·6 mL fractions were collected for analysis. —— Eluted by buffer, - - - - eluted by $10^{-4}$ M tenoxicam.

*Proton titration experiments*

Fig. 2 shows the titration curves of HSA (pH vs $Z_H$, see eqn 2), which are in close agreement with previously published ones (Janssen et al 1981). The standard deviation in these curves (n = 3) was between 0·2 and 1·7%.

The number of protons released from the HSA molecule differs according to the presence or absence of ligands. Considering the respective dissociation of diazepam (a weak base of $pK_a$ 2·15), tenoxicam (an acid of $pK_a$ 5·3) and warfarin (an acid of $pK_a$ 5·05), negligible proton release occurs from these ligands when the pH rises above 6. Thus, the observed proton release is due to HSA. When the anionic ligands tenoxicam (1 mol mol$^{-1}$ HSA) or warfarin (1 mol mol$^{-1}$ HSA) were added, proton release was markedly increased (Fig. 2A, B). In contrast, diazepam binding (1 mol mol$^{-1}$ HSA) was accompanied by decreasing proton release (Fig. 2C). Differences were seen for the three drugs when

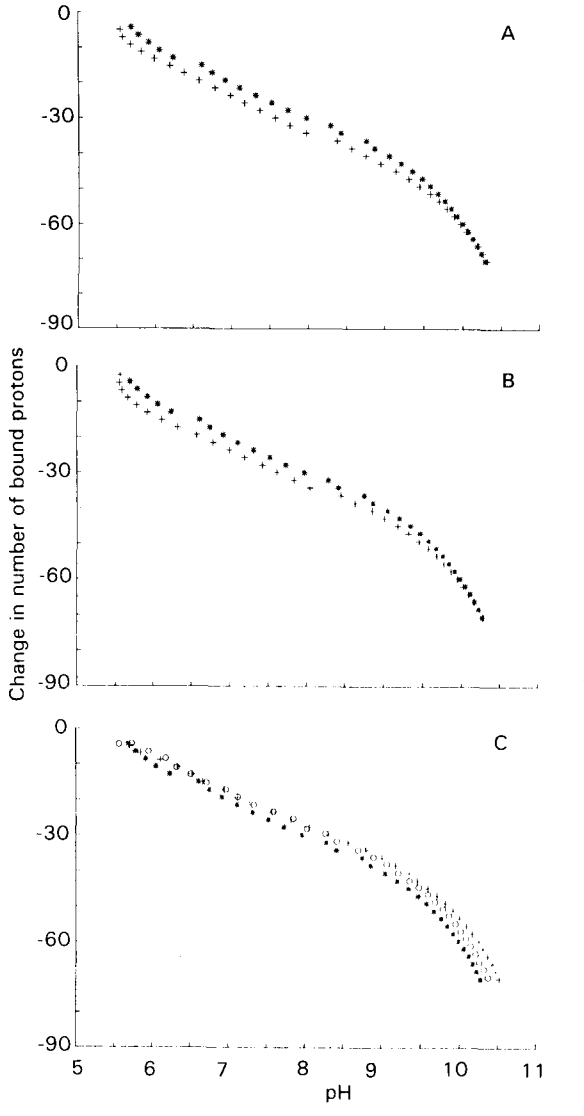


FIG. 2. Titration curves of A, HSA with tenoxicam (+, 1:1), B, HSA with warfarin (+, 1:1), and C, HSA with diazepam (○, 1:1) and HSA with diazepam and tenoxicam (+, 1:1:1). * Denotes the titration curve of HSA alone (150 μM in 0·15 μM KCl).

Table 2. Binding equilibrium constants ($10^6$ $M^{-1}$) of tenoxicam to HSA at 25 and 15°C with and without diazepam.

| | Buffer | | $10^{-4}$ M diazepam | |
|---|---|---|---|---|
| | $K_{a1}$ | $K_{a2}$ | $K_{a1}$ | $K_{a2}$ |
| At 25°C | | | | |
| pH 6·8 | 0·251 | 0·062 | — | — |
| pH 7·4 | 0·368 | 0·074 | 0·535+ | 0·033 |
| pH 9·2 | 0·795* | 0·079 | 0·899 | 0·043 |
| At 15°C | | | | |
| pH 6·8 | 0·412* | 0·073 | — | — |
| pH 7·4 | 0·621 | 0·084 | 1·116+ | 0·051 |
| pH 9·2 | 1·300* | 0·100 | 1·633+ | 0·047 |

Equilibrium dialysis, phosphate buffer (pH 6·8 and 7·4) or borate buffer (pH 9·2). *$P < 0·05$ compared with value at pH 7·4. + $P < 0·05$ compared with corresponding value without diazepam.

changing the ligand/HSA ratios, but these differences were modest and too close to standard deviations to be interpretable.

Thus, ligand binding to site I is accompanied by proton release, suggesting the increased deprotonation of one or more acidic groups (e.g. OH or $NH^+$). Site II binding appears accompanied by reduced deprotonation of one or more acidic groups on HSA. We have recently shown that binding of warfarin to $\alpha_1$-acid glycoprotein is accompanied by an increase in the basicity of an imidazolyl group in the protein ($pK_a$ shift from 7·7 to 8·9) (Urien et al 1993).

Of interest is also the proton release in the ternary complex HSA-tenoxicam-diazepam (Fig. 2C). The curve is superimposable on that of the binary complex HSA diazepam in the pH range 5·5–8. At higher pH values, tenoxicam potentiates the reduction in deprotonation elicited by diazepam, suggesting that tenoxicam increases the binding of diazepam. This is fully consistent with the results from affinity chromatography (Table 1).

*Equilibrium dialysis measurements*

We have observed previously (Brée et al 1989) that two main interactions occur between HSA (10 μM) and tenoxicam (0·5–50 μM); the first one is of high affinity and corresponds to the binding of tenoxicam to the warfarin area of HSA, while the second one is of lower affinity and involves the binding of tenoxicam to the benzodiazepine site. Results presented in Table 2 show that increasing the pH from 6·8 to 7·4 to 9·2 (N to B transition) produced a marked increase in the high-affinity binding, whereas there was only a negligible effect on the second affinity constant.

When $10^{-4}$ M diazepam (i.e. diazepam is ten times the concentration of HSA) was added, tenoxicam binding to site I increased while a slight decrease was observed for binding to site II. These two phenomena are more pronounced at 15°C than at 25°C. Previous reports have described conformational changes of the HSA molecule induced by various ligands including drugs (Birkett et al 1977; Sjöholm et al 1979). The possibility of allosteric effects was also suggested (Brée et al 1989) since palmitic acid increased tenoxicam binding to the site I of HSA at low palmitic acid to HSA ratio and decreased tenoxicam binding at higher ratios as for other site II ligands (Fehske et al 1981; Wanwimolruk et al 1983). The present data clearly indicate that, when the pH increases from 6·8 to 9·2, the strength of the tenoxicam-HSA interaction increases. As rising pH increases the negative charges on the protein, a decrease of the ability to attract anions might be expected. We have observed that tenoxicam increases proton release, thus increasing the number of negative charges on the protein which should lead to an additional decrease in tenoxicam binding. However, the opposite effect is observed as tenoxicam binding is reinforced. This seems to rule out an electrostatic drug-ligand interaction and suggests that the pH-dependent conformational change of HSA is induced by electrostatic forces within the protein and results in a tighter binding of tenoxicam.

Diazepam was seen to increase the high-affinity binding of tenoxicam (Tables 1, 2, Fig. 1), this effect being accompanied by a potentiated inhibition in the release of protons (Fig. 2C). Hence diazepam, by stabilizing the N state, prevents proton release and limits the reduction of negative charges.

This, in turn, should diminish the repulsion between the two anions HSA and tenoxicam, suggesting that diazepam might increase tenoxicam binding by favouring electrostatic interactions.

It seems, therefore, likely that upon binding, tenoxicam and diazepam induce two different changes in HSA. Tenoxicam alters the conformation at or around site I as does a pH increase, resulting in its tighter fit and higher affinity. Diazepam, on the other hand, prevents proton release and this may account for a potentiated binding of tenoxicam. Such a facilitated binding at the warfarin area in the presence of certain benzodiazepines was also demonstrated by Fitos et al (1986) and an allosteric interaction between the two binding sites was similarly suggested by Dröge et al (1985), using microcalorimetry. The facilitated effect seems specific for diazepam and not for all benzodiazepines, since lorazepam (a weak acid) did not increase tenoxicam binding (results not shown).

The findings and interpretations reported here confirm that allosteric interactions can exist between ligands binding respectively to site I and II of HSA. Indeed, both tenoxicam (site I) and diazepam (site II) increase each other's binding, but they appear to do so by somewhat different mechanisms. While diazepam may increase tenoxicam binding by favouring electrostatic interactions, a similar effect cannot explain the increased binding of diazepam caused by tenoxicam; in this case, it appears that tenoxicam acts by inducing conformational changes that increase both its own binding to site I and that of diazepam to site II.

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