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Technical Note

Binding of Piroxicam to Human Serum Albumin: Effect of Piroxicam on Warfarin and Diazepam Binding

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INTRODUCTION

The interaction of drugs and a wide variety of endogenous substances such as bilirubin, hormones, and fatty acids with human serum albumin (HSA) has been extensively studied.

One of the physicochemical properties of human and bovine serum albumin is the drastic conformational change around neutral pH (1–4). This conformational change is now commonly referred as neutral-to-base or N–B transition. Recently, Ca^{2+} and Cl^- ions have been shown to affect this N–B transition (5,6).

Until recently, no workers had shown the involvement of the N–B transition in drug–human serum albumin (HSA) interactions. The first work reported was with warfarin (6). According to the report, the drug has an increased affinity for HSA in the B form. Ca^{2+} ions also increase the affinity of the drug for HSA, presumably by altering the conformation, but the Cl^- ions, at high concentrations, decrease the affinity.

Later, a paper on the diazepam–HSA interaction was published, which again showed an increased affinity for the drug when HSA is in the B form; however, Ca^{2+} ions decreased the affinity of the drug for HSA (7). Subsequently, other drugs such as benoxaprofen (8) have also been cited in support of N–B transition in drug–HSA interactions.

The binding of the nonsteroidal antiinflammatory drug piroxicam [4-hydroxy-2-methyl-*N*-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide] to HSA has been investigated under physiological conditions (9–11). In this paper, the effect of pH, Ca^{2+}, and Cl^- ions on the binding of piroxicam to HSA has been investigated. Also, the effect of piroxicam on warfarin and diazepam binding has been observed using displacement studies.

MATERIALS AND METHODS

Human serum albumin (HSA), fraction V (lot No. 35F-9442), was obtained from Sigma Chemical Company, St. Louis, Mo. Piroxicam was a gift of the Pfizer Corp., Groton, Conn. Diazepam (lot No. 380121) was donated by Hoffman LaRoche, Nutley, N.J. Sodium warfarin (lot No. 71-167-1) was obtained from Endo Laboratories Inc., Garden City, N.Y. Solutions of albumin in deionized water were deionized by passing through a mixed-bed ion-exchange column (Dowex 50W-X8 and 1 × 8 as previously described). The molr ratio of free fatty acid to HSA in the original sample (1.9) was reduced to 0.8 after deionization. The fatty acid content was determined by the method of Chen (12).

Equilibrium dialysis experiments were performed (6) with a Dianorm equilibrium dialyzer (Diachema A.G. Ruschlikon, Switzerland) using cells of 1.0-ml total volume. Hydrated cellulose membranes (Diachema, type 10.15, molecular weight cutoff of 5000) were washed with deionized water and dried with a tissue paper. Adsorption of piroxicam onto these membranes was negligible, and the volumes of the solutions on either side of the membrane stayed constant during the dialysis procedure. After the 6-hr dialysis at 37°C, free concentrations of piroxicam, diazepam, and warfarin were determined by high-pressure liquid chromatography using a Zorbax ODS (4.6-mm × 15-cm) column in a Varian 5000 liquid chromatograph, detection being effected by a Specroflow 757 absorbance detector (Kratos, Ramsey, N.Y.) at 237 nm. A mobile phase of 35% acetonitrile in deionized water (pH 5.2 with 0.05 *M* phosphate buffer) was used for piroxicam, diazepam, and warfarin. Both the drug and the protein used were in their pure form; as a result, no problem was encountered from interfering peaks.

RESULTS AND DISCUSSION

A Scatchard plot of the dialysis data using both nondeionized and deionized HSA at pH 7.4 and 37°C is shown in Fig. 1. The data were analyzed assuming two independent classes of binding sites, using the computer program described by Perrin *et al.* (13). For deionized HSA the best fit was obtained with the binding data $n_1 = 1$, $K_1 = 1.6 \pm 0.5 \times 10^5\ M^{-1}$ and $n_2 = 1.5$, $K_2 = 3.04 \pm 0.5 \times 10^3\ M^{-1}$ (n_1 and n_2 being kept fixed).

Schiantarelli *et al.* (14) reported a value of K_1 an order of magnitude higher, and K_2 had the impossible value of $10^7\ M^{-1}$. These discrepancies may be due to differing fatty acid

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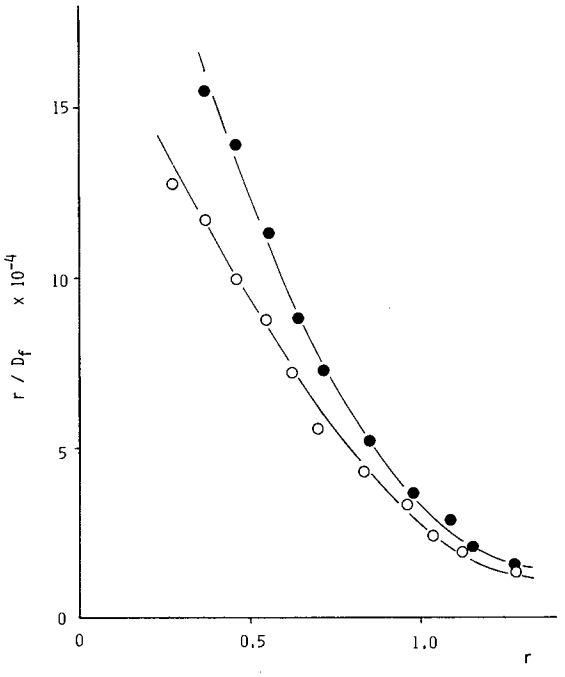

Fig. 1. Scatchard plot of the dialysis data for the binding of piroxicam to deionized HSA (○) and nondeionized HSA (●) at pH 7.4 and 37°C.

contents of the albumins. Several cases of fatty acids changing the degree of binding of drugs to HSA have been reported: warfarin by oleic acid (15), tolmetin by oleic acid (16), and penicillin by palmitic acid (17). The primary association constant for piroxicam was significantly higher with increased fatty acid content of the HSA (nondeionized; Fig. 1), the binding parameters changing to $K_1 = 2.4 \times 10^5\ M^{-1}$, $n_1 = 1.0$ and $K_2 = 3.2 \times 10^3\ M^{-1}$, $n_2 = 1.5$. This suggests a significant effect of the fatty acids on the binding of piroxicam to HSA, and this is probably the reason for the discrepancies observed in the literature. The commercial samples of albumin have been found to have fatty acid-to-HSA ratios varying from 0.03 to 9.0 (18,19). No standard procedure for determining the fatty acid content was used in the literature investigations of the piroxicam–HSA interaction.

The primary association constant (K) increases from 0.90 to $2.3 \times 10^5\ M^{-1}$ as the pH increases from 6.0 to 9.0. In the pH region 6.0 to 9.0 no changes in the physicochemical properties of piroxicam are known to occur, and the observed pH dependence should have its origin in the albumin molecule.

Zurawski and Foster (20) showed evidence for the existence of two distinguishable states of HSA in this pH range. These states have been reported (2,3,5) to be influenced by the physiologically important Ca^{2+} and Cl^- ions. In spite of a large number of reports dealing with the pH dependence on the affinity of drugs and inorganic ions to HSA, there is as yet not much information regarding the relationship between the conformational change of the albumin molecule and the binding parameters of drugs.

In order to support such a linkage between the N–B transition in HSA and the affinity of piroxicam for HSA, the effect of Ca^{2+} and Cl^- ions on the affinity of piroxicam for HSA was also examined, as well as the effect of H^+ ions.

Table I. Free Concentration of Piroxicam Determined Following Equilibrium Dialysis in the Presence of Warfarin or Diazepam at pH 7.4 in 0.1 M Phosphate Buffer at 37°C[a]

Displacing agent	Conc. of displacing agent ($\times 10^4\ M$)	Free piroxicam ($\times 10^6\ M$)
Warfarin	None	4.54 ± 0.23
	1.25	5.48 ± 0.38*
Diazepam	None	4.28 ± 0.23
	1.25	4.24 ± 0.11 (NS)[b]

[a] HSA = $2.5 \times 10^4\ M$; total piroxicam = $1.25 \times 10^{-4}\ M$.
[b] No significant difference.
* Significant difference compared with none ($P < 0.02$ by two tailed t test).

The 0.1 M chloride solution decreased the association constant of piroxicam from $1.9 \pm 0.08 \times 10^5\ M^{-1}$ in the control (pH 7.4, 0.1 M phosphate buffer) to $1.2 \pm 0.06 \times 10^5\ M^{-1}$. However, $10^{-2}\ M$ calcium increased it from $1.1 \pm 0.03 \times 10^5\ M^{-1}$ in Tris–HCl buffer to $1.6 \pm 0.03 \times 10^5\ M^{-1}$. Cl^- ions at high concentrations compete with the drug for the binding site in the same way as they did for warfarin binding site (6). On the other hand, CA^{2+} ions increased the warfarin–HSA complexes (6), but for diazepam–HSA complexes (7) they caused a reduction in affinity. Judging from the present results on the effect of Ca^{2+}, Cl^-, and H^+ ions on the binding of piroxicam to HSA, the binding behavior of piroxicam is quite similar to that of warfarin.

Displacement studies for piroxicam using the two representative drugs, diazepam and warfarn, were also performed by the dialysis technique. Tables I and II show that warfarin increases the free concentration of piroxicam from $4.54 \pm 0.23 \times 10^{-6}$ to $5.48 \pm .038 \times 10^{-6}\ M$, whereas diazepam did not increase the free concentration at the same low drug-to-protein ratios. On the other hand, a mutual displacement study demonstrates that piroxicam increases not only free concentrations of warfarin but also the free concentration of diazepam. Possibly, the diazepam binding site,

Table II. Free Concentration of Warfarin and Diazepam Determined Following Equilibrium Dialysis with or Without Piroxicam (Displacer) at pH 7.4 in 0.1 M Phosphate Buffer at 37°C[a]

Displacing agent	Conc. of displacing agent ($\times 10^4\ M$)	Free concentration ($\times 10^6\ M$)
		Free warfarin
Piroxicam	None	6.70 ± 0.38
	1.25	8.82 ± 0.29**
		Free Diazepam
Piroxicam	None	6.9 ± 0.30
	1.25	8.5 ± 0.25*

[a] HSA = $2.5 \times 10^{-4}\ M$; total warfarin = $12.5 \times 10^{-4}\ M$; total diazepam = $1.25 \times 10^{-4}\ M$.
* Significant difference compared with none ($P < 0.02$ by two-tailed t test).
** Significant difference compared with none ($P < 0.01$ by two-tailed t test).

site 2, is susceptible to the allosteric changes induced by the binding of piroxicam to HSA. In contrast, Zini *et al.* (21) reported that warfarin was displaced by indomethacin, while indomethacin was not displaced by warfarin. The authors claimed that indomethacin induced a modification of the warfarin binding site, site 1, of albumin. The present experiments emphasize that mutual displacement experiments in which both free concentrations are determined as essential for classification of the primary binding site.

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