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Plasma protein binding and interaction studies with piroxicam

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Summary. The binding of non-steroidal antirheumatic drug piroxicam to human serum albumin, human plasma and serum has been studied by equilibrium dialysis at 22° C, pH 7.4. The binding data were analyzed according to Scatchard model. The values of binding parameters obtained for human serum albumin are quite similar to those obtained for human plasma and serum ($N_1 = 0.3$, $K_1 = 3.0 \cdot 10^5$ l/mol; $N_2 = 7$, $K_2 = 3.5 \cdot 10^3$ l/mol). We suggest that piroxicam interacts with the albumin fraction in human plasma proteins.

The displacement of piroxicam (in the therapeutical concentration of $4.5 \cdot 10^5$ mol/l) from the binding to human serum albumin and human plasma has been studied. The concentration of albumin and albumin fraction in plasma was $2.9 \cdot 10^{-4}$ mol/l. The displacement substances were drugs – diazepam, warfarin and salicylic acid, and endogenous substances-bilirubin and palmitic acid. Only in the presence of salicylic acid in high clinical concentration ($14.5 \cdot 10^{-4}$ mol/l) and palmitic acid in the molar ratio to albumin 5:1, free piroxicam substantially increased, which may be of clinical significance. Other studied substances displaced piroxicam only in high concentrations exceeding the therapeutical and physiological range.

The evidence was found for the similarity of piroxicam and warfarin high-affinity binding site.

Key words: Piroxicam – Protein binding – Displacement – Binding site

Introduction

The non-steroidal antiinflammatory drug piroxicam is structurally unrelated to other compounds with these properties. Clinical studies showed that piroxicam possesses a long half time 38–45 h (Hobbs and Twomey 1979; Ishizaki et al. 1979; Hobbs 1983). The long half time might suggest either strong protein binding or efficient extent of enterohepatic recycling.

The information in the literature about the binding of piroxicam to human plasma protein is not satisfactory. Hobbs and Twomey (1971) described the piroxicam binding to human serum albumin only in per cent (98–99%). Schiantarelli et al. (1981) indicated binding constants for the binding of piroxicam to diluted plasma (1:10 with phosphate buffer) to have the values: $N_1 = 1$, $K_1 = 4.57 \cdot 10^6$ l/mol; $N_2 = 2$, $K_2 = 2.27 \cdot 10^7$ l/mol. Quite different values for binding parameters were obtained by Rendić et al. (1981). At the human serum albumin concentration of $1.45 \cdot 10^{-4}$ mol/l, they found values for $N_1 = 1.1$, $K_1 = 1.96 \cdot 10^5$ l/mol; and for $N_2 = 2.9$, $K_2 = 0.154 \cdot 10^5$ l/mol. They studied also the displacement of piroxicam by diazepam and desmethyldiazepam, but only in one concentration $1.45 \cdot 10^{-5}$ mol/l.

The fact that there are conflicting results about the binding characteristics of piroxicam and very little information about the displacement of bound piroxicam by drugs and endogenous substances, led us to present investigation of the binding of piroxicam to human serum albumin, human plasma and serum. Further was studied the displacement of bound piroxicam with drugs-diazepam, warfarin and salicyclic acid and with endogenous substances – bilirubin and palmitic acid.

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Material and Methods

Materials. Human serum albumin fraction V (HSA) and human serum albumin, essentially free of fatty acids (HSA-FAF) were purchased from Sigma Company, St. Louis, MO, USA. Plasma obtained from the transfusion station was kept at −20° C.

4-Hydroxy-2-methyl-N-/2-pyridyl/-2H-1,2-benzothiazine-3-carboxamide 1,1 dioxide-piroxicam (Felden) was a gift of Pfizer Corp., Groton, CT, USA, 3-(acetonylbenzyl)-4-hydroxycoumarin-warfarin, bilirubin, palmitic acid and salicylic acid were obtained from Sigma Company, St. Louis, MO, USA. Diazepam was a gift from Research Institute of Pharmacy and Biochemistry, Prague.

Equilibrium dialysis. The binding of piroxicam to HSA and plasma was determined by equilibrium dialysis, 20 h at 22° C, using a HSA concentration of $2.9 \cdot 10^{-4}$ mol/l and albumin concentration in plasma and serum $2.9 \cdot 10^{-4}$ mol/l. All solutions were prepared with phosphate buffer pH 7.4 ionic strength 0.111. HSA and plasma were applied to one side of the membrane (Visking) and piroxicam, alone or together with studied substances, to the other side. Substances not soluble in phosphate buffer were first dissolved in a small volume of dimethyl sulphoxide (in a final concentration of 1%). One milliliter dialysis cells were closed and kept for 20 h, shaken occasionally. Experimentally we have found that this time was sufficient to reach the equilibrium. Preliminary experiments showed no absorption onto the cell or the membrane and no dimethyl sulphoxide effect. The increase in plasma free fatty acids (FFA) level occurring during equilibrium dialysis referred by Ridd et al. (1982),

was in our conditions about 10%. Molar ratio of plasma albumin fraction to FFA was 1:0.74 before dialysis and 1:0.89 after dialysis. In this molar ratio the inhibition of piroxicam binding did not occur (see Results). Effect of temperature on the binding of piroxicam to HSA was not significant. Percentage of binding at 22°C was 93.5 ± 0.17 and at 37°C it was 92.7 ± 0.14% (at piroxicam concentration $3 \cdot 10^{-5}$ mol/l and HSA concentration $2.9 \cdot 10^{-4}$ mol/l). Duplicate samples of HSA, plasma and serum were used at each drug concentration.

Analytical methods. Free concentration of piroxicam in buffer solutions was assayed according to Ishizaki et al. (1979). The concentration of free fatty acids (FFA) was determined according to Novák (1965). The albumin concentration in plasma and serum was determined by fluorescence method according to Udenfried (1962).

Determination of binding parameters. The analysis of the protein binding data obtained was based upon the assumption that the binding of piroxicam occurred to albumin. Scatchard plots of the data were analyzed assuming of two classes of binding sites, with the number of binding sites. N_1, N_2 and the association constants K_1, K_2 (Scatchard 1949). These parameters were estimated at first by graphic method of Rosenthal (1967) and the best estimation was obtained by means of computerized least-squares fitting of Scatchard model to the experimental binding data, minimizing the error function. The curve-fitting procedure, using the nonlinear least-squares program NONLIN (Metzler 1969), was based upon the following equation:

$$v/D_f = NK - Kv$$

where v is a number of piroxicam-bound molecules per mol of albumin, D_f is a molar concentration of free piroxicam. All computations were done on Hewlett-Packard-85 computer. The binding data obtained from in vitro displacement studies were plotted in a double reciprocal way (Klotz and Hunston 1979). Linear regression was performed to determine the best line through the experimental points.

In the text and tables the results are presented as mean ± SD.

Results

The binding of piroxicam to pure HSA, human plasma and serum

The binding of piroxicam over the range $3-150 \cdot 10^{-5}$ mol/l was studied at a HSA concentration $2.9 \cdot 10^{-4}$ mol/l. The concentration of albumin fraction in human plasma and serum was $2.9 \cdot 10^{-4}$ mol/l. The binding percentages decreased from 94–74% when piroxicam concentration raised from $3-150 \cdot 10^{-5}$ mol/l.

Figure 1 shows the Scatchard curves for the binding of piroxicam to HSA, human plasma and serum. The heterogenity of binding sites is expressed by the non-linearity of the Scatchard plot. The mean binding parameters resulting from the analysis of these data are summarized in Table 1. The number of primary and secondary binding sites and the values of association constants are similar for all three proteins, there are no differences between binding of piroxicam to HSA, to human plasma and serum

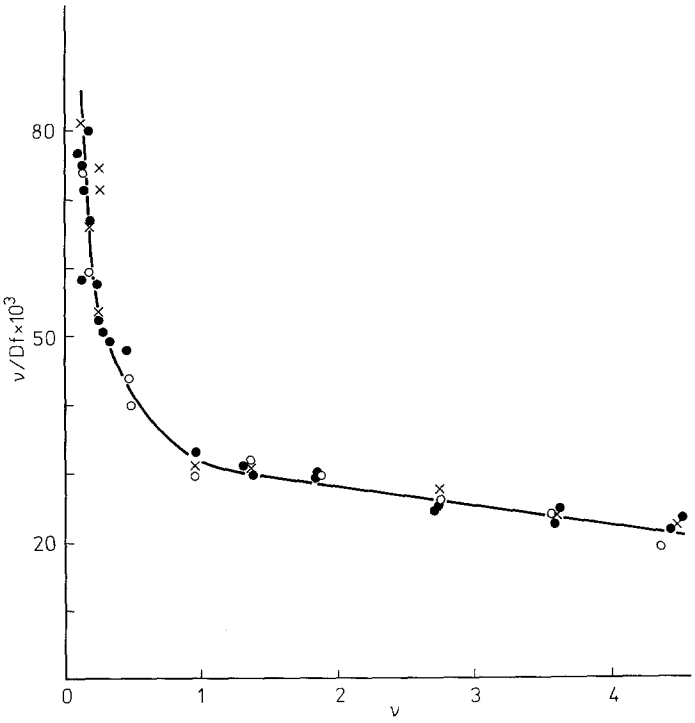

Fig. 1. Scatchard plot for the binding of piroxicam to pure HSA ○——○, human plasma ●——● and human serum ×——×. Each point represent the mean value of the least three experiments. v = number of piroxicam bound per mole of albumin, D_f = molar concentration of free piroxicam

Table 1. Binding characteristics of piroxicam to human serum albumin, human plasma and serum

Protein	N_1	$K_1 \cdot 10^5$ l/mol	N_2	$K_2 \cdot 10^3$ l/mol
HSA	0.33 ± 0.02	3.00 ± 0.26	7.60 ± 0.30	3.95 ± 0.45
Plasma	0.30 ± 0.01	3.03 ± 0.24	7.80 ± 0.41	3.85 ± 0.23
Serum	0.32 ± 0.04	3.28 ± 0.35	7.85 ± 0.48	3.18 ± 0.52

HSA concentration = $2.9 \cdot 10^{-4}$ mol/l; albumin fraction in plasma and serum = $2.9 \cdot 10^{-4}$ mol/l; data are means ± SD of six determinations

Effect of drugs and endogenous substances on the in vitro piroxicam binding to HSA

In the present work three drugs – diazepam, warfarin and salicylic acid and two endogenous substances – bilirubin and palmitic acid have been used as displacing agents. We determined the free concentration of piroxicam at a constant low molar ratio of HSA: piroxicam = 1:0.15. This concentration of piroxicam ($4.5 \cdot 10^{-5}$ mol/l) is within the therapeutic range (Ishizaki et al. 1979). The concentration of HSA ($2.9 \cdot 10^{-4}$ mol/l) corresponds closely to the physiological concentration of albumin present in human plasma in rheumatic and some other diseases (Tillement et al. 1978). The results are presented in Table 2. A marked increase in free piroxicam was observed when bilirubin was present in the molar concentration to HSA = 1:1. The same effect had warfarin in the molar ratio to HSA = 3:1 and palmitic acid in the high molar ratio 5:1. Mildest displacing effect was observed in the presence of diazepam and salicylic acid in the molar ratio to HSA 3:1. In the experiments with palmitic acid we have used HSA-FAF:

Table 2. The free concentration of piroxicam determined following equilibrium dialysis in the presence of displacing agents

Displacing agent	Concentration of displacing agent mol/l · 10^{-4}	Molar ratio of HSA: displacing agent	Molar ratio of displ. a.: pirox.	Unbound piroxicam mol/l · 10^{-6}	Unbound piroxicam %
Diazepam	none			4.973	11.0
	0.45	1:0.15	1: 1	5.513	12.2
	2.9	1:1	1: 6.4	7.063	15.6
	8.7	1:3	1:19.3	7.919	17.6
Warfarin	none			5.141	11.4
	0.45	1:0.15	1: 1	5.537	12.3
	2.9	1:1	1: 6.4	6.525	14.5
	8.7	1:3	1:19.3	9.489	21.1
Salicylic acid	none			4.637	10.3
	0.45	1:0.15	1: 1	4.832	10.7
	2.9	1:1	1: 6.4	5.030	11.8
	8.7	1:3	1:19.3	6.802	15.1
Bilirubin	none			4.940	11.0
	0.45	1:0.15	1: 1	5.723	12.7
	2.9	1:1	1: 6.4	10.630	23.6
Palmitic acid[a]	none			4.787	10.6
	0.45	1:0.15	1:1	5.571	12.3
	2.9	1:1	1:6.4	5.766	12.8
	8.7	1:3	1:19.3	5.961	13.2
	14.5	1:5	1:32.2	10.65	23.7

The concentration of HSA = 2.9 · 10^{-4} mol/l, the concentration of piroxicam = 4.5 · 10^{-5} mol/l, molar ratio HSA:piroxicam = 1:0.15
[a] HSA-FAF was used at the concentration 2.9 · 10^{-4} mol/l; data are means of five determinations

Table 3. Therapeutic and physiological levels and binding constants of displacing substances

Displacing substance	Conc. range mol/l	Reference	Used. conc. mol/l	K_1 l/mol	N_1	Reference
Diazepam	0.7— 2.1 · 10^{-6}	Wilting et al. (1980)	1.4 · 10^{-6}	2.0 · 10^5	1	Wilting et al. (1980)
Warfarin	3.2—32.4 · 10^{-6}	Vesel et al. (1975)	32.4 · 10^{-6}	2.3 · 10^5	1	Tillement et al. (1974)
Salicylic acid	3.6—21.7 · 10^{-4}	Levy (1979)	7.2 · 10^{-4} 14.5 · 10^{-4}	1.3 · 10^5	2	Hultmark et al. (1975)
Bilirubin	0.5— 2.0 · 10^{-5}	Jacobsen (1969	2.0 · 10^{-5}	1.4 · 10^8	1	Jacobsen (1969)
Palmitic acid	2.0—3.5 · 10^{-4} (non-fasting adults) 5.0—9.0 · 10^{-4} (fasting adults)	Ridd et al. (1982)	2.9 · 10^{-4} 8.7 · 10^{-4}	6.0 · 10^7	2	Goodman (1958)

Effect of clinical and physiological concentration of displacing agents on the binding of piroxicam to plasma protein

For the evaluation of clinical importance, the displacement interactions were made with human plasma, using the therapeutic steady-state concentrations of drugs and physiological concentrations of endogenous substances (Table 3). The binding data obtained were plotted in a double reciprocal way. This approach was used to determine the nature of the displacement for only low, clinically relevant piroxicam concentration data.

Salicylic acid in the concentration 14.5 · 10^{-4} mol/l inhibits the binding of piroxicam to plasma protein (Fig. 2a). Diazepam and warfarin, in the therapeutic level concentration have no effect (Fig. 2b). Bilirubin binds very strongly to albumin. Adding bilirubin in the physiological concentration (2.0 · 10^{-5} mol/l) to plasma, however, did not influence the plasma protein binding of piroxicam (Fig. 3a). Free fatty acids are physiological substances which have high affinity to serum albumin. Double reciprocal plots in Fig. 3b demonstrate an inhibition effect of palmitic acid on the primary binding sites of piroxicam only in the high concentration of 8.7 · 10^{-4} mol/l.

Discussion

Results of the present investigations show a high affinity of piroxicam to HSA and plasma protein. The primary association constant for the binding of piroxicam to HSA is

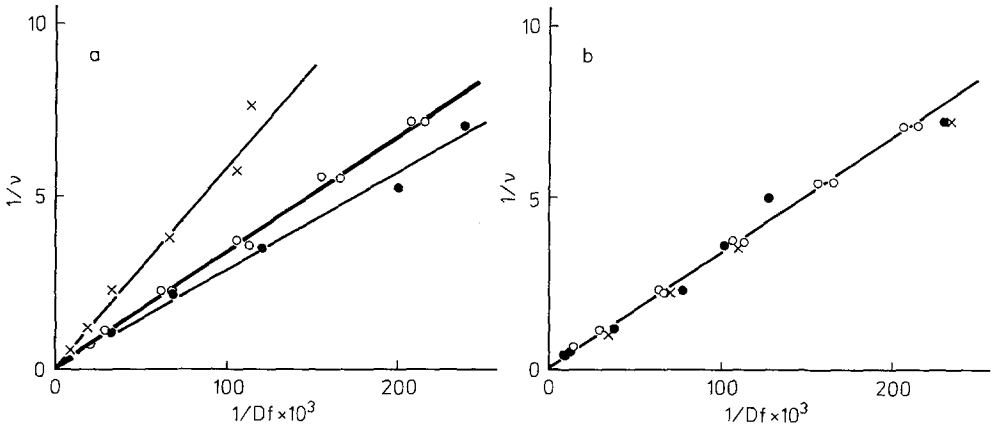

Fig. 2a, b
Double reciprocal plot illustrating the effect of salicylic acid, warfarine and diazepam on the primary binding sites of piroxicam to plasma protein. **a** ○——○ control, ●——● salicylic acid $7.2 \cdot 10^{-4}$ mol/l, ×——× salicylic acid $14.5 \cdot 10^{-4}$ mol/l; **b** ○——○ control, ●——● warfarin $32.4 \cdot 10^{-6}$ mol/l, ×——× diazepam $1.4 \cdot 10^{-6}$ mol/l. The concentration of albumin fraction in plasma was $2.9 \cdot 10^{-4}$ l/mol

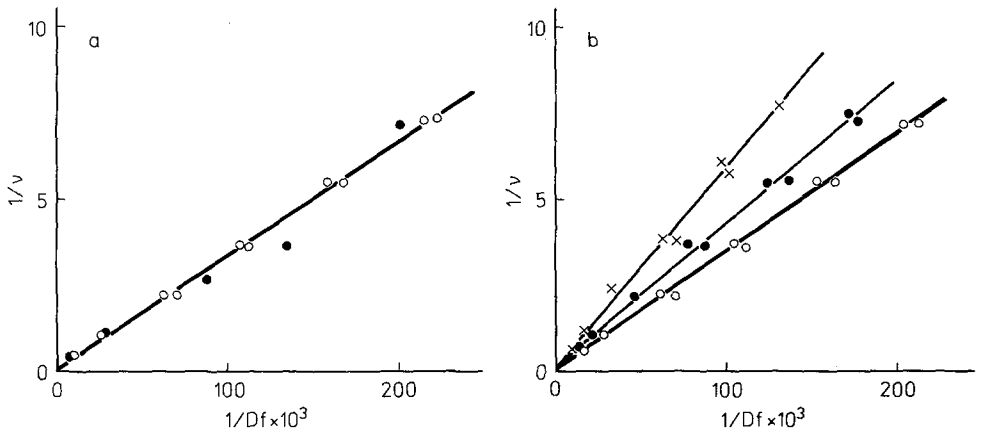

Fig. 3a, b
Double reciprocal plot illustrating the effect of bilirubin and palmitic acid on the primary binding sites of piroxicam to plasma protein. **a** ○——○ control, ●——● bilirubin $2.0 \cdot 10^{-5}$ mol/l; **b** ○——○ control, ●——● palmitic acid $2.9 \cdot 10^{4}$ mol/l; ×——× palmitic acid $8.7 \cdot 10^{-4}$ mol/l. The concentration of albumin fraction in plasma was $2.9 \cdot 10^{-4}$ mol/l

$3.0 \cdot 10^5$ l/mol. For human plasma and serum we have found $3.03 \cdot 10^5$ l/mol and $3.28 \cdot 10^5$ l/mol, respectively. In view of the comparable piroxicam binding to HSA and plasma, we suggest that piroxicam primarily interacts with the albumin fraction in human plasma proteins.

The clinical use of a new drug which is extensively bound to plasma protein requires consideration of possible interaction with the protein binding of other substances. The increase of unbound drug may enhance the effect of the drug and alter its distribution and elimination (Wilkinson 1983). We have studied the displacing effect of three drugs – diazepam, warfarin and salicylic acid and two endogenous substances – bilirubin and palmitic acid in various molar ratios to piroxicam and to HSA. It was found that on the molecule of albumin a few high-affinity sites and a variable number of different low-affinity sites are specifically involved and are located and characterized (Krach-Hansen 1981; Fehske et al. 1981; Sjöholm et al. 1979). Diazepam, warfarin, bilirubin and palmitic acid are typical and fairly selective ligands for high-affinity binding sites on albumin molecule. Adding displacing agents in molar ratio to piroxicam 1:1 did not influence the piroxicam binding to HSA. In the presence of three moles of warfarin per mole of HSA, free piroxicam increased in about a half. We can suggest that high affinity binding site of piroxicam is identical with warfarin site. Bilirubin at a molar ratio of one to HSA strongly reduces the binding of piroxicam, which is typical for drugs binding to warfarin (Fehske et al. 1981). Palmitic acid reduced substantially the binding of piroxicam in the high molar ratio to HSA 5:1. This is in agreement with numerous studies on the interaction between fatty acids and drugs (Rudmann et al. 1971; Solomon et al. 1968; Tsutsumi et al. 1975; Chakrabarti 1978). It was shown that at high concentrations (at a molar ratio of 4–7 to albumin) free fatty acids have a inhibitory effect on the binding of many drugs.

The importance of drug displacement interaction, particularly those at plasma albumin sites, has been very often overestimated (McElnay and D'Arcy 1983). Therefore, we measured also the binding of piroxicam (it the clinical concentration $4.5 \cdot 10^{-5}$ mol/l) to human plasma (with the concentration of albumin fraction $2.9 \cdot 10^{-4}$ mol/l) in the presence of displacing agents in adequate therapeutical and physiological concentrations. The binding of piroxicam was substantially reduced by palmitic acid at the concentration $8.7 \cdot 10^{-4}$ mol/l, which is the highest physiological level in fasting adults. Also salicylic acid, at concentration $14.5 \cdot 10^{-4}$ mol/l has the displacing effect on the piroxicam plasma protein binding. This is a much higher concentration than that of piroxicam, but is typical for clinical situation. Hobbs (1983) has reported that the coadministration of aspirin reduces piroxicam plasma levels by about 20% in a group of twenty volunteers. Displacement effect of salicylate was observed also by warfarin (Aarons et al. 1979).

During therapeutical use of piroxicam it is necessary to consider the displacing effect of salicylic acid and free fatty acids, but only in high concentration levels.

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