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COMMUNICATIONS

The energetics of the interaction of piroxicam with plasma albumin

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The thermodynamics of the binding of piroxicam to human and bovine plasma albumins revealed the reactions to be spontaneous and exothermic with bond strengths indicating the involvement of hydrogen and hydrophobic bonds. The association constants (K) decreased as temperature increased for both human serum albumin (HSA) and bovine serum albumin (BSA); and at all the temperatures in this study, K values obtained for BSA were higher (except at 36 °C) but not the bound fraction of piroxicam (β) and the quantity of binding (V). Scatchard plots of the data indicated at least two classes of binding sites.

Piroxicam (4-hydroxy-2-methyl-*N*-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide) is a non-steroidal anti-inflammatory drug (NSAID) currently marketed for rheumatoid arthritis (Ishizaki et al 1979). Preliminary disposition studies in animals (Wiseman 1977) and man (Wiseman 1977; Nuotio & Mäkisara 1977) have suggested that its pharmacokinetic profile may be different from those of other NSAIDs recently available (Brogden et al 1977) and its half life appears to be long, 1·4–1·6 days (Ishizaki et al 1979) in man. Biotransformation studies have shown that it is largely metabolized since only 10% of a dose is excreted in the urine unchanged and its distribution is such that after a single oral dose of 30–60 mg, maximal plasma concentrations are maintained for 120–168 h (Ishizaki et al 1979). In-vivo studies with human plasma show piroxicam to be strongly protein bound at a concentration close to clinical values (Mäkisara & Nuotio 1978).

The nature and type of reactions involved when piroxicam binds to albumin and the effects of temperature on the affinity of the drug for the binding sites have been examined.

Materials and methods

Materials. Piroxicam was the generous gift of Pfizer (Nig) Ltd., Lagos, Nigeria and was of the highest available purity. The solutions of drugs were made by diluting a stock solution with a concentration of 10 mM in 0·067 M sodium phosphate buffer, pH 7·4.

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Human serum albumin (HSA; Sigma USA) and bovine serum albumin (BSA; BDH, Poole, UK) were used. The protein solutions used in the dialysis studies were made at a concentration of 0·4 mg ml^{-1} (5·8 × 10^{-6} M) in 0·067 M sodium phosphate buffer, pH 7·4 (ionic strength (I) = 0·17).

Dialysis. Binding was studied by equilibrium dialysis. Visking dialysis tubing lengths (10 cm × 3 cm o.d.) (Scientific Instrument Centre, Ltd, London) were washed to remove associated impurities, one end tied and the lengths then stored in phosphate buffer solution at 4 °C.

Binding measurements were made as follows: 5 cm^3 of albumin solution placed inside the tubing was dialysed against 10 cm^3 of medium containing piroxicam in tightly capped glass tubes to avoid evaporation. Six concentrations (1·5, 3·0, 7·5, 15·0, 22·5, 30·0 × 10^{-5} M) of drug were used with protein at 5·8 × 10^{-6} M. For each experiment, six tubes of each drug concentration were rocked at 160 min^{-1} in a water bath at 5·0 ± 1 °C, 20·0 ± 2 °C or 36·0 ± 1 °C for 18 h, when equilibrium would have been attained, and aliquots of drug solution were removed for determination of free drug.

Adsorption of drug to the Visking membranes was usually small, about 1·2%, and this figure was used to correct ligand concentration (A).

Analytical techniques. Measurement of the maximum uv absorption was made in the region of 340 nm, at which, peak absorption occurred. Linear dependence of optical density was observed with all concentrations. The concentration of free drug was estimated by measuring the absorbance at wavelength 355 nm.

Evaluation of binding data. All the binding data were plotted according to the Scatchard (1949) method. A molecular weight of 69 000 was assumed for both human and bovine serum albumin. In the Scatchard plot, deviations from linearity occur when there is more than one class of binding site or when the binding sites do not satisfy the demand on independency and/or equivalency. Assuming the existence of two classes of binding sites, the Scatchard equation may be written as

$$V = \{n_1K_1[A]/1 + K_1[A]\} + \{n_2K_2[A]/1 + K_2[A]\}$$

where V, the degree or quantity of binding, is the molar ratio of bound drug to total protein: [A] is the molar concentration of the corrected free ligand at equilibrium; K_1 and K_2 are the site association (binding) constants, and n_1 and n_2 are the average maximal number of binding sites in the respective classes of binding sites.

K_1 and K_2 are evaluated graphically by fitting the data into a linear regression analysis and obtaining the slopes and intercepts of the extrapolated tangents drawn at the extremities of the plots (Klotz & Huntson 1971) on the assumption of non-interaction between the classes of binding sites.

Evaluation of thermodynamic binding. The nature of the binding process for piroxicam and proteins can be analysed by the thermodynamic relationship $\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ$ where ΔF° is the standard free energy change on binding, ΔH° is the standard enthalpy change on binding, T is the absolute temperature and ΔS° is the standard entropy change on binding. ΔF° was calculated from K using the general thermodynamic relationship: $\Delta F^\circ = -RT \ln K\ \Delta H^\circ$, the temperature dependent part of the interaction, was calculated from the van t-Hoff equation

$$\ln \frac{K_2}{K_1} = \frac{\Delta H(T_2 - T_1)}{RT_2T_1}$$

Results

Table 1 shows the calculated parameters of the binding of piroxicam to HSA and BSA and gives the initial ligand concentration (A), extent of binding (β) and the quantity or degree of binding (V) at 5, 20 and 36 °C. Table 2 gives the thermodynamic data and the association constants at the various temperatures. Figs 1 and 2 show the Scatchard profiles of the interaction of piroxicam with the proteins in 0·067 M phosphate buffer (pH 7·4) at the temperatures used.

The extent of piroxicam binding to HSA and BSA is similar at low drug concentrations ($0{\cdot}15 \times 10^{-4}$ M), being 90% at 5 °C and a protein concentration of $5{\cdot}8 \times 10^{-6}$ M (Table 1).

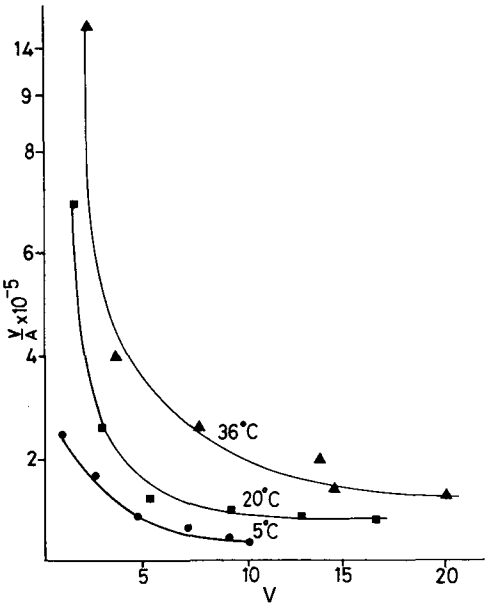

FIG. 1. Scatchard plots illustrating the binding of piroxicam to HSA (0·4 mg ml^{-1}) at 5, 20 and 36 °C. Regression lines fitted to the data for piroxicam concentrations 30×10^{-5} M and below.

Although the extent and the degree of binding of piroxicam to HSA were similar to that of BSA at the same temperature and ligand concentration (concentrations above $0{\cdot}15 \times 10^{-4}$ M), the K values for BSA were higher (Table 2), the K_1 values ranging from 2·89 to $13{\cdot}23 \times 10^{-5}$ litre mol^{-1} for BSA and 3·16 to $11{\cdot}5 \times 10^{-5}$ litre mol^{-1} for HSA. Values were highest at the lowest temperatures and vice versa. The K_2 values ranged from 0·56 to $2{\cdot}23 \times 10^{-5}$ litre mol^{-1} for BSA and 0·79 to $2{\cdot}05 \times 10^{-5}$ litre mol^{-1} for HSA.

Table 1. Binding data for piroxicam showing the extent of binding (β) and the quantity of binding (V) at 5, 20 and 36 °C at different concentrations of the ligand.

Initial ligand concn [A] × 10^{-5} M	Binding parameters	5 °C		20 °C		36 °C	
		HSA	BSA	HSA	BSA	HSA	BSA
1·5	β	89·0 ± 3·5	90·0 ± 2·5	80·0 ± 1·5	80·0 ± 1·5	65·0 ± 1·5	65·5 ± 1·0
	V	2·3 ± 0·1	2·3 ± 0·1	2·1 ± 0·2	2·2 ± 0·9	1·5 ± 0·1	1·6 ± 0·1
3·0	β	70·5 ± 2·5	75·0 ± 2·0	60·5 ± 2·0	65·0 ± 2·0	50·0 ± 1·5	55·0 ± 2·0
	V	3·6 ± 0·4	3·9 ± 0·2	3·4 ± 0·3	3·4 ± 0–5	2·6 ± 0·2	2·8 ± 0·2
7·5	β	66·0 ± 2·5	68·0 ± 2·5	44·5 ± 1–75	44·0 ± 1·5	36·0 ± 1·5	40·5 ± 2·0
	V	7·8 ± 1·0	8·8 ± 0·9	5·2 ± 0·6	5·9 ± 0·8	4·7 ± 0·8	5·2 ± 0·6
15·0	β	54·0 ± 1·75	56·0 ± 1·5	36·0 ± 1·5	40·5 ± 1·0	29·0 ± 1·0	32·0 ± 1·5
	V	13·9 ± 0·8	14·5 ± 0·8	9·3 ± 1·1	11·1 ± 1·2	7·2 ± 0·7	7·8 ± 0·8
22·5	β	44·0 ± 1·5	45·0 ± 2·0	34·0 ± 1·0	37·5 ± 1·0	24·0 ± 1·0	28·0 ± 1·0
	V	14·5 ± 1·6	17·9 ± 1·4	12·9 ± 1·2	14·5 ± 1·0	9·3 ± 0·9	12·9 ± 0·8
30·0	β	40·0 ± 1·5	42·0 ± 1·5	32·5 ± 1·0	35·0 ± 1·5	20·0 ± 1·0	25·0 ± 2·0
	V	21·0 ± 1·2	21·7 ± 1·4	16·6 ± 1·8	10·3 ± 1·6	10·3 ± 1·1	13·4 ± 0·8

Values given for mean of 5 experiments (±s.e.m.) with six determinations in each experiment.

When the binding of piroxicam to BSA and HSA was studied at different temperatures and at different ligand concentrations but constant protein concentration, the extent of binding decreased with increasing temperature and decreased with increasing ligand concentration (Table 1).

Transformation of the binding data into Scatchard plots results in non-linear profiles with negative slopes, the profiles being curvilinear and bending sharply towards the abscissa (Figs 1, 2) suggesting the existence of multiple number and classes of binding sites.

Binding data as in Figs 1, 2 were analysed by fitting the Scatchard equation to the experimental points by minimization of least square differences of observed and expected values (the latter being omitted for clarity).

The thermodynamic data in Table 2 are for the first class of binding site and indicate that there is an inverse relationship between the binding strength and the experimental temperature.

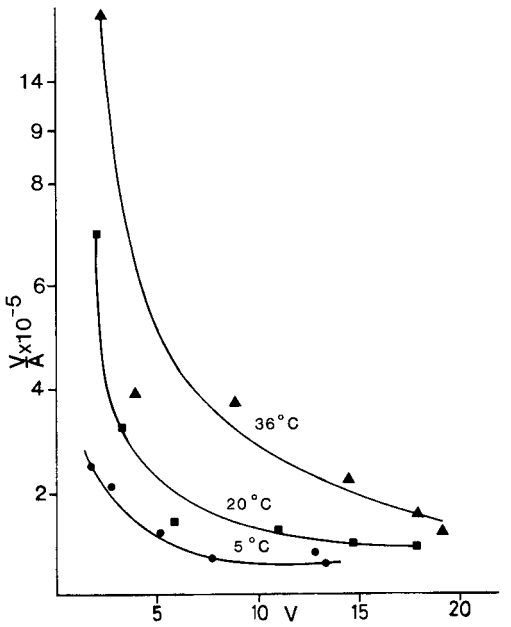

FIG. 2. Scatchard plots illustrating the binding of piroxicam to BSA (0·4 mg ml^{-1}) at 5, 20 and 36 °C. Regression lines fitted to the data for piroxicam concentrations 30 × 10^{-5} M and below.

Discussion

The data from our study and the binding curves from BSA and HSA results were similar at all temperatures. They were all non-linear indicating that more than one class of binding site was involved in the binding of piroxicam to the proteins. One group was of high affinity (K_1) and low capacity (n_1) and the other of low affinity (K_2) and correspondingly high capacity (n_2).

Table 2. Binding data for piroxicam with serum albumins at 5, 20 and 36 °C.

Association constants (litres mol^{-1}) × 10^{-5}	BSA			HSA		
	36 °C	20 °C	5 °C	36 °C	20 °C	5 °C
K_1	2·89	6·12	13·23	3·16	5·74	11·50
$Log_{10} K_1$	5·46	5·79	6·12	5·50	5·76	6·06
K_2	0·56	1·13	2·23	0·79	1·15	2·05
$Log_{10} K_2$	4·75	5·05	5·35	4·90	5·06	5·31
Standard free energy $\Delta F°$ (kcal mol^{-1})	−7·71	−7·75	−7·77	−7·76	−7·71	−7·70
(kJ mol^{-1})	32·3	32·4	32·5	32·5	32·3	32·3
Standard enthalpy change $\Delta H°$ (kcal mol^{-1})	−8·42	−8·30	−8·30	−6·70	−7·48	−7·48
(kJ mol^{-1})	35·2	34·7	34·7	28·0	31·3	31·3
Standard entropy change $\Delta S°$ (Gibbs mol^{-1})	−2·31	−1·90	−1·91	+3·44	+0·76	+0·76

All thermodynamic data are for the first class of binding site only.

Binding studies with piroxicam were carried out and analysed thermodynamically to determine the nature and type of reactions, including their temperature dependence. Under our experimental conditions, the interactions between piroxicam and albumin were of the spontaneous and exothermic type as indicated by the negative sign of $\Delta H°$. At equilibrium the relationship, [D + P $\rightleftharpoons$ DP + heat] shows the free drug (D) and protein (P) in a reversible relationship with the drug-protein complex (DP), the reaction being accompanied by evolution of heat. A decrease in temperature will favour the forward reaction, as evidenced by an increased percentage of the bound drug as experimental temperature decreases (Table 1).

The low entropy change at all temperatures, coupled with negative $\Delta F°$, indicates that the energy forces involved in the protein-piroxicam interaction are being produced within the system, hence it is definitely spontaneous. The large contribution to $\Delta F°$ by $\Delta H°$ indicates that binding energy is from non-ionic sources and so from the data presented, it may be suggested that hydrogen bonding is mainly involved, with some contribution from hydrophobic bonding, and this is in agreement with previous findings (O'Reilly 1973; Bezkorovainy 1963).

The values for K were lowest at the highest temperatures with a significant increase at lower temperatures. At 5 °C, the values were increased three to four fold for both BSA and HSA. The reduction in binding strength with increased temperature is characteristic of exothermic reactions as reported for albumin binding of warfarin, thyroxine, methicillin and oxacillin and for hapten-antibody and insulin-antibody reactions (O'Reilly 1969, 1973).

The bond strength of the protein-piroxicam interaction may be accounted for by $\Delta F°$ values averaging −7·7 Kcal mol^{-1} (−32 kJ mol^{-1}) at all the temperatures used. The significant evolution of heat also suggests hydrogen bonding in the absence of any significant

electrostatic interaction. The ΔS changes probably result from hydrophobic binding (O'Reilly 1969).

If the binding of piroxicam involves both hydrogen and hydrophobic bonds at multiple binding sites, we suggest that the drug binds strongly and that the dose required might be such that only a relatively small amount will be available in plasma to achieve this therapeutic effect. It is therefore not surprising that the amount of piroxicam in plasma following a single oral dose of 20 mg produces a good therapeutic effect at plasma level as low as 5 $\mu g\ ml^{-1}$ (Mäkisara & Nuotio 1978) compared with other drugs used for arthritis. In addition, the high protein binding nature of the drug in human plasma has been documented and was confirmed by our observation (Table 2) where about 90% of the drug was bound to the albumins at $0{\cdot}15 \times 10^{-5}$ M at 5 °C.

From Table 1 the progressive reduction in the fraction of piroxicam bound as its free concentration increases (with the amount of protein unchanged) reflects the saturable nature of the binding sites by the drug at high concentrations.

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Inhibition of lipid absorption by non-ionic hydrophobic surfactant Pluronic L-81, and its benzoyl esters

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The benzoyl ester of Pluronic L-81 (BEP) has been purified on an aluminium oxide column and fractionated on the basis of its mobility characteristics into three fractions, BEP I, II and III. Absorption studies were made with rats fed by gavage a lipid preparation containing [^{14}C]triolein and [^{3}H]cholesterol (control animals) and one of the fractions or Pluronic L-81, or crude BEP. After 4 h the amount of lipid absorbed and transported was calculated as the difference between the dose fed and the amount of lipid recovered from the gastric and intestinal luminal contents and intestinal mucosa. Pluronic L-81 was the most effective in inhibiting absorption of triolein but it did not inhibit absorption of cholesterol. BEP-II was almost as effective in inhibiting the absorption of triolein and also inhibited absorption of cholesterol. Crude BEP was less effective and BEP-I and III had only limited activity. Inhibition of triolein absorption by Pluronic L-81 may be partly related to its delaying action on gastric emptying. As purified BEP preparations had practically no effect on gastric emptying, their inhibiting activity involved intestinal mechanisms of lipid absorption.

We have previously shown that the benzoyl ester (BEP) of the hydrophobic poloxalene, Pluronic L-81, is an effective hypolipaemic agent comparable in activity to the parent compound but less toxic and much better tolerated by laboratory animals (Kapuscinska et al 1982b; Bochenek & Rodgers 1977). Used on a chronic basis in rabbits fed atherogenic diet, BEP decreased serum cholesterol (Kapuscinska et al 1982a). The mechanism by which BEP exerts its hypolipaemic effect is thought to be the same as other hydrophobic surfactants composed of polypropylene oxide and polyethylene oxide which during absorption of fat cause an accumulation of lipid in the intestinal mucosal cells (Brunelle et al 1979; Bochenek et al 1983). This was shown to be associated with decreased secretion of chylomicrons into intestinal lymph (Tso et al 1981). The observed effects are readily reversible in as little as 30 min after discontinuation of surfactant (Bochenek et al 1983).

BEP does not have a uniform structure and experiments have been designed to examine the activities of crude BEP and fractions derived from it and compare these with Pluronic L-81 in absorption studies of lipids in rats.

Materials and methods

Radioactive chemicals, glycerol tri [1-^{14}C] oleate and [1,2(n)-^{3}H] cholesterol were purchased from Amersham Corp., Arlington Heights, IL, and were purified by thin layer chromatography (tlc) before use. Triolein and cholesterol were from ICN Pharmaceuticals, Cleve-

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