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SHORT COMMUNICATIONS

Comparative warfarin binding to albumin from various species

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Variations in drug binding to plasma proteins among species may determine differences in pharmacologic potency, drug distribution and elimination rates [1]. Differences in species binding for diazoxide [2], digitalis glycosides [3], dipenylhydantoin [4], imipramine [5], salicylates [6] and sulfonamides [7] have been reported. There is considerable variation in the anticoagulant effect of coumarins among animal species [8–10]. The human, rat and mouse are most sensitive; guinea pigs, cats and dogs intermediate; and the rabbit, cow and chicken least affected. To what extent these variations can be accounted for by differences in free plasma water coumarin concentrations is not known. To determine the extent of variation, we have studied the binding of warfarin to albumins from eight species.

The binding of warfarin (Endo Laboratories, Garden City, NY) to albumins from various species was studied by equilibrium dialysis [11, 12]. Albumin solutions (approximately 1 g/100 ml) (Pentex Corp., Kankakee, IL) were prepared from non-defatted purified fraction V albumins. The calculations of binding isotherms were based on the actual albumin concentration in the protein solution of each species. Binding was assumed to be only to albumin. All experiments were done at 37° in 0.067 M, phosphate buffer, pH 7.4. Three replicates for each of eight different warfarin concentrations (0.6, 0.8, 1.0, 1.5, 2.0, 3.0, 5.0, and 7.0×10^{-6} M) were studied. Each equilibrium dialysis tube consisted of 2.0 ml of protein solution (approximately 0.3 μmole albumin) contained in the dialysis tubing immersed in 10.0 ml of buffered drug solution (maximum 0.007 μmole drug).

Experiments were done to determine the binding isotherm for rac-warfarin to the test tube glass and dialysis tubing. At free warfarin concentrations of 1×10^{-6} M, approximately 2×10^{-10} moles of drug were lost to these sites. This represents <0.1 per cent of the total warfarin added initially and no corrections for loss of drug to these sites were applied. Binding results were analyzed by fitting a logistic function [11, 12].

$$E = M \cdot (D)^P / [(D)^P + (K)^P]$$

to the experimental points by a minimization of least squares differences of observed and expected values (E = moles of drug bound/mole of albumin, D = free drug concentration). This mathematical form indicates a curve with a maximum M, a position K along the abscissa at $M/2$ and a slope determined by the power P. The computer program first fits N curves independently to N sets of binding results, then tests the parallelism amongst curves and finally tests for differences in position between any two parallel curves, with the variance ratio test [11]. This technique provides an objective curve fitting procedure, incorporates a statistical test of differences and presents plotted data in an easily understood fashion.

The best fit lines for the binding of rac-warfarin to rat, human, sheep and dog albumin are shown in Fig. 1. At all free warfarin concentrations, rat albumin binds warfarin more than the other three albumins. A Scatchard plot yields identical conclusions, but the extent of the differences and relation to free warfarin concentration are not as readily appreciated. Figure 2 presents only the fitted lines for the experimental results and shows the ratio of the number of moles of warfarin bound/mole of albumin for each non-human albumin compared to the number of moles of warfarin bound to human albumin. This representation emphasizes that the relative binding of warfarin to other albumins changes as a function of free drug concentration and that the binding curves are not parallel to the isotherm for human albumin. Table 1 summarizes the values for the calculated parameters P and K and gives the assigned value for M. All binding isotherms differ significantly from that for warfarin binding to human albumin. Other studies with whole serum show that warfarin binding characteristics for dog and rabbit are different from the purified albumin binding, since binding is greater to whole serum (Table 1).

There is marked inter-species variation in warfarin binding. Rat albumin binds more warfarin than human, whereas binding to horse albumin is minimal. The lesser binding to canine albumin is in accord with a previous report [13]. Since the binding isotherms are not parallel (Fig. 2), the number of apparent sites participating in binding are different among albumins. The extent of these qualitative and quantitative differences in binding among species is frequently ignored [1–8]. In fact, free drug concentrations in plasma water are measured so seldom that the importance of species variation in binding among and within species is not known. In humans, free diazepam

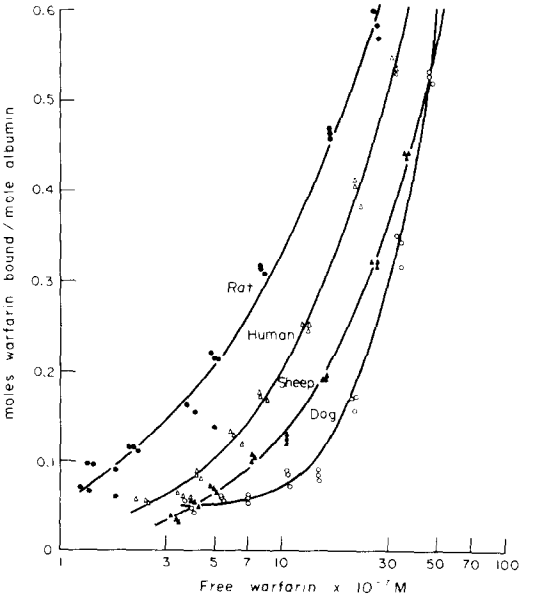

Fig. 1. Binding of warfarin to albumin from four different species.

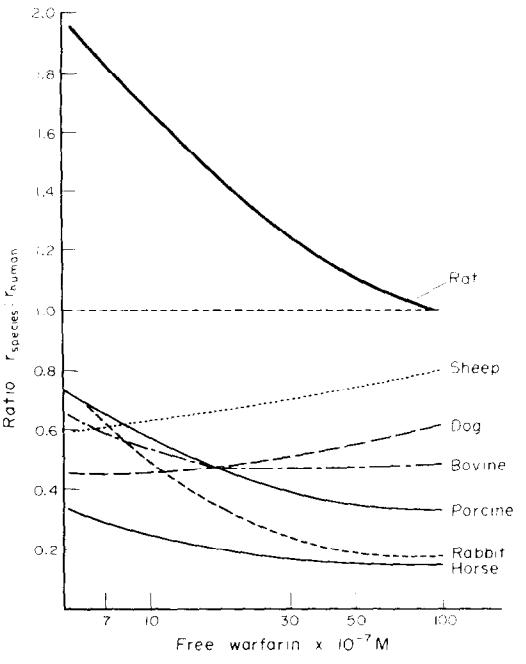

Fig. 2. Relative binding of warfarin to human and non-human albumins. For the ratio, r = moles warfarin bound/mole of albumin.

concentrations vary considerably among individuals with identical serum albumin concentrations [14]. The extent of nortriptyline binding to serum albumin varies considerably among individuals and is under genetic influence [5, 15].

The data in Fig. 2 show not only that binding is dependent on free warfarin concentration but also that the binding isotherms are not necessarily symmetrical or parallel among species. Hence, experiments that fail to study binding over a wide range of free drug concentrations can result in divergent estimates of the numbers of binding sites or affinity. Entirely contradictory results could occur when isotherms of two different species binding intersect. Free drug concentrations less than the point of intersection would predict greater binding for the albumin that would give lower binding at concentrations greater than the point of intersection. *In vivo*, drug dose–effect curves in two different species might not be parallel because of differences in albumin binding.

If free warfarin concentration were the major determinant of drug potency, then rabbit, horse and pig with least binding should be most sensitive to the drug. However, there is no apparent inverse relationship between degree of albumin binding and anticoagulant sensitivity [8–10]. In fact, the species with the most extensive binding (human and rat) are most sensitive, suggesting that differences in affinity at the site of action among species and not free warfarin concentration account for differences in response. There is considerable other evidence that receptor affinity is the most important determinant of anticoagulant response to coumarins. Evidence for this can be deduced from studies of human kindreds that require 10-fold greater doses of warfarin for a therapeutic effect [16]. The S-isomer of warfarin is most highly bound to human albumin [12], yet it is more potent and more rapidly metabolized than the R-isomer, suggesting that differences in potency of the two isomers are due primarily to differences in "receptor" site affinity and not differences in albumin binding [17]. The inverse relationship of rate of metabolism and potency with free drug concentration is particularly interesting if shown to be true for other highly bound drugs, e.g. benzodiazepines, since this would imply that receptor affinity differences account for inter-individual differences and that free drug concentration measurements may correlate poorly with pharmacologic response except within the same individual. More widespread measurement of free drug concentrations will be necessary to determine whether warfarin is unique in this respect. Recently published studies indicate that free propranolol concentrations correlate significantly better than does propranolol dose, total concentration or red cell concentration with degree of β-receptor blockade [18].

An alternate explanation for "greater sensitivity" with greater binding is that the "reservoir function" of an albumin-bound drug leads to slower elimination and in the case of oral anticoagulants a more prolonged inhibition of vitamin K clotting factor synthesis [1]. Therefore, for

Table 1. Calculated binding parameters*

	Maximum (M)	Slope (P)	Position (K) $\times 10^{-6}$ M	Variance slope	Ratio position
Albumin					
Rat	2	0.77	8.24	8.1†	3.9‡
Human	2	1.02	8.7		
Sheep	2	1.07	0.124	4.0‡	7.9†
Dog	2	1.02	0.193	3.4‡	10.1§
Bovine	2	0.81	0.351	4.7‖	12.9§
Porcine	2	0.56	0.0149	8.2†	13.1§
Rabbit	1	0.29	0.00249	14.8§	18.5§
Horse	2	0.54	0.0849	8.5†	18.8§
Serum					
Dog	2	1.57	9.26		
Cat	2	1.23	0.145		
Rabbit	2	1.41	0.136		

* Degrees of freedom for slope (1,22) and position (1,23). Comparisons are to human albumin. See text for description of binding parameters.
† $P < 0.01$.
‡ Not significant.
§ $P < 0.005$.
‖ $P < 0.05$.

some drugs difference in serum protein binding and free drug concentration may account for variation within and among species.

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Interactions between phosphatidylcholines and trihexyphenidyl and benztropine

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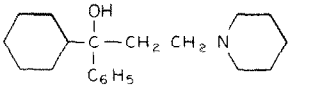
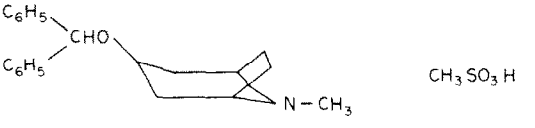
In principle, drugs can block the action potential in nerve by affecting any of the conductance parameters that control the nerve excitability. In practice, many local anaesthetics block the action potential by blocking the sodium current. The major problem in accounting for this effect is that it is surprisingly unselective, with many neutral, negatively charged and positively charged molecules acting as local anaesthetics [1]. This can be explained if the site of action of the anaesthetics is the lipid component of the nerve membrane, but it is then necessary to explain the connection between action on lipids and the blockage of sodium current. A general increase in fluidity will not do, since any changes in fluidity for the bulk lipids of the membrane will be insignificant at the relevant drug concentrations [2, 3]. A more specific effect has to be postulated, as in the annular transition model [4]. In this model, the sodium channels in nerve membranes are postulated to be surrounded by lipid molecules in a rigid or gel-like state. Addition of local anaesthetics triggers a change in the surrounding lipids to a fluid or liquid crystalline state, allowing the sodium channel to relax to an inactive configuration, in which the sodium current is reduced or blocked.

In previous publications it has been shown that for alcohols [5], amines [6], barbiturates [7], chlorpromazine [3] and β-blockers [3] the concentrations required to produce blocking of the sodium current in nerve also cause a decrease of ca 3° in the temperatures of the gel to liquid crystalline phase transition in phospholipids. In order to extend the correlation to further classes of compounds, the effects of the anti-Parkinsonian drugs, trihexyphenidyl (I) and benztropine (II), on the phase transition temperatures of lipids have now been studied. In a recent paper Wu and Narahashi [8] have shown that these compounds act as local anaesthetics, blocking the sodium current.



Trihexyphenidyl hydrochloride (I)



Benztropine mesylate (II)

The temperatures of the gel to liquid crystalline phase transitions in dipalmitoyl phosphatidylcholine were measured using chlorophyll *a* as a fluorescence probe, as described elsewhere [5]. Liposomes were prepared from lipid (6×10^{-7} moles) in 4 ml 0.01 M Tris–HCl containing 0.1 M NaCl at a final pH 7.2, the anaesthetic being first dissolved at the required concentration in the buffer. The anaesthetics were obtained as trihexyphenidylhydrochloride and benztropine mesylate.