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THE EFFECT OF VARIOUS DRUGS ON THE BINDING OF WARFARIN-^{14}C TO HUMAN ALBUMIN*

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Abstract—The effect of various drugs on the binding of warfarin-^{14}C to human albumin was studied by the technique of ultrafiltration. Phenylbutazone, *d*-thyroxine, sulfaphenazole, and chlorophenoxyisobutyric acid (CPIB) were less effective than unlabeled warfarin in displacing warfarin-^{14}C from albumin. The organic bases, phenyramidol and atropine, had little displacing effect. Phenylbutazone competitively inhibits the binding of warfarin-^{14}C to albumin. CPIB binds to a different site on albumin than does warfarin-^{14}C and noncompetitively inhibits the binding of the anticoagulant. Both phenylbutazone and CPIB, at concentrations observed clinically, increased the unbound fraction of warfarin-^{14}C 10-fold and 4-fold respectively. Phenyramidol and *d*-thyroxine had no effect on the binding of warfarin-^{14}C under similar circumstances.

Decreased binding of coumarin-type drugs to albumin produced by the administration of other drugs may cause an increased anticoagulant effect in clinical situations.

MANY DRUGS are extensively bound to plasma proteins; it is generally accepted that the bound fraction is devoid of pharmacologic activity.[1] Displacement of a drug from its binding site on albumin by other drugs may cause an increase in its pharmacologic effect.[2, 3]

Although it is known that warfarin and *bis* hydroxycoumarin are extensively bound to albumin,[4, 5] no studies of the displacement of these anticoagulants from their binding sites by other drugs have been reported.

Since several drugs are known to increase the anticoagulant response to coumarin-type compounds,[6–8] the present study was undertaken to characterize the effect of a number of such drugs on the binding of warfarin to human albumin.

METHODS

Human albumin (chromatographically isolated, 96 per cent pure) and *d*-thyroxine were obtained from the Mann Research Laboratories. Other drugs were obtained as follows: atropine sulfate from K & K Laboratories; chlorophenoxyisobutyric acid (CPIB) from Ayerst Laboratories; phenylbutazone from Geigy Pharmaceuticals; sulfaphenazole (Orisul) from CIBA Pharmaceutical Co.; and phenyramidol from Neisler Laboratories, Inc. Dr. Collin H. Schroeder of the Wisconsin Alumni Research Foundation generously provided samples of warfarin and of warfarin-^{14}C. The radioactive compound was labeled in the 4-position of the coumarin moiety and had a sp. act. of 5·9 mc/m-mole.

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Human albumin, atropine, phenyramidol, sulfaphenazole, warfarin, and warfarin-^{14}C were dissolved in a potassium dihydrogen phosphate–disodium hydrogen phosphate buffer, ionic strength 0·1 and pH 7·4. Phenylbutazone, *d*-thyroxine, and CPIB were dissolved in 0·1 N NaOH.

The ultrafiltration technique of Rehberg[9] was used to determine the extent of binding of warfarin-^{14}C to albumin. The dialysis bags were prepared from cellulose tubing, 5/8 in. in diameter. Each bag contained 0·5 ml of albumin solution, 0·1 ml of warfarin-^{14}C (0·1 μc), 0·1 ml of drug solution, and 4·3 ml of the buffer. In control experiments the drug solution was omitted and 4·4 ml of buffer was added. The bags were centrifuged in 50-ml polypropylene tubes at 6000 rpm for 60 min at 30°.

One tenth ml of the ultrafiltrate and 0·1 ml of the contents of each dialysis bag were added to 10 ml of *p*-dioxane containing 0·05 g POPOP [1,4-bis-2(5-phenyoxazoyl)benzene], 7·0 g PPO (2,5-diphenyloxazole), and 50 g naphthalene/l. Warfarin-^{14}C in the medium was determined with a Packard Tri-Carb spectrometer. Counts per minute were at least 20 times background.

Preliminary experiments demonstrated that there was no binding of warfarin-^{14}C to the dialysis membrane.

The formulation of Klotz[10] was used to estimate the number of binding sites for warfarin on human albumin and to determine the magnitude of the affinity of warfarin for the protein. In certain studies the extent of binding of warfarin at various concentrations was measured in the presence of a fixed concentration of either phenylbutazone or CPIB.

RESULTS

A. Binding of warfarin-^{14}C at various concentrations of albumin

The extent of binding of warfarin-^{14}C to human albumin increased markedly as the albumin concentration was increased from 0 to 0·03 mM. At an albumin concentration of 0·15 mM, approximately 94 per cent of the drug was bound (Fig. 1).

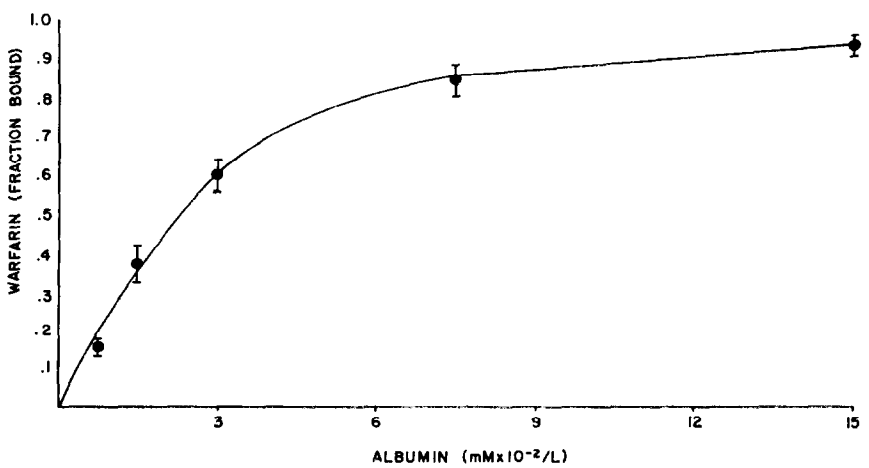

FIG. 1. The relation between the concentration of human albumin and the extent of binding of warfarin-^{14}C. In all experiments the final concentration of warfarin-^{14}C was $3{\cdot}3 \times 10^{-3}$ m-mole. Each point is the mean ± S.D. of four determinations.

B. The effect of various drugs on the binding of warfarin-^{14}C to human albumin (0·15 *m-mole*)

Many acidic drugs displace warfarin-^{14}C from human albumin (Table 1). Unlabeled warfarin, at a concentration 60 times that of the labeled compound, reduced

TABLE 1. EFFECT OF DRUGS ON THE BINDING OF WARFARIN-^{14}C TO HUMAN ALBUMIN

Drug	mM	m-mole Drug / m-mole Warfarin-^{14}C	Warfarin-^{14}C (% bound)
Warfarin	0·20	60	28·3 ± 2·6
Phenylbutazone	0·66	200	28·7 ± 3·3
d-Thyroxine	1·16	350	29·5 ± 1·4
Sulfaphenazole	4·62	1400	27·8 ± 2·2
Chlorophenoxyisobutyric acid	6·60	2000	26·7 ± 2·3
Phenyramidol	3·30	1000	78·1 ± 1·5
Atropine	3·30	1000	9·26 ± 92·6

In all experiments the final concentration of warfarin-^{14}C was $3{\cdot}3 \times 10^{-3}$ m-mole, and the final concentration of albumin was 0·15 m-mole. In control experiments warfarin-^{14}C was 93·7 ± 1·1 per cent bound. Results are the mean ± S.D. of 4–8 determinations.

the binding of warfarin-^{14}C from 93·7 per cent to approximately 28 per cent. Other drugs were less effective than unlabeled warfarin in displacing warfarin-^{14}C from albumin. Approximately 3 times more phenylbutazone, 6 times more *d*-thyroxine, 25 times more sulfaphenazole, and 30 times more CPIB than unlabeled warfarin were required to displace warfarin-^{14}C to the same extent.

Basic compounds were much less effective than acids in displacing warfarin-^{14}C from albumin. For example, atropine, at a concentration 1000 times that of labeled warfarin, did not alter the binding of warfarin-^{14}C to albumin. Phenyramidol, at the same concentration, reduced the binding of warfarin-^{14}C to only 78 per cent.

C. The nature of the displacement of warfarin-^{14}C from human albumin by phenylbutazone and CPIB

There was a linear relationship between the reciprocal of the concentration of unbound warfarin-^{14}C and the reciprocal of the moles of warfarin-^{14}C bound per mole of albumin over the range of concentrations of warfarin-^{14}C studied (Figs. 2 and 3). Warfarin-^{14}C binds to a single site on human albumin with an affinity constant of 84,000.

In the presence of phenylbutazone, less warfarin-^{14}C was bound to albumin (Fig. 2). A common ordinate intercept indicates that warfarin-^{14}C and phenylbutazone compete for the same binding site on albumin. The inhibitory constant (K_i) of phenylbutazone against the binding of warfarin-^{14}C by human albumin was estimated from $K_i = i/(Kp/K - 1)$, where i equals the concentration of inhibitor, and Kp is the apparent affinity constant (K) in the presence of the inhibitor. In the present study, K_i for phenylbutazone was 165,000.

Less warfarin-^{14}C was bound to albumin in the presence of CPIB (Fig. 3). The lack of a common ordinate intercept indicates that warfarin-^{14}C and CPIB do not compete for the same site on the protein.

D. Effect of clinical concentrations of phenylbutazone, CPIB, phenyramidol, and d-*thyroxine on the binding of warfarin-*^{14}*C to human albumin*

Concentrations of phenylbutazone,[11] CPIB,[12] warfarin,[5] and albumin based on commonly observed clinical values were selected. As the concentration of phenylbutazone increased from 0 to 150 μg/ml, the binding of warfarin-^{14}C decreased from

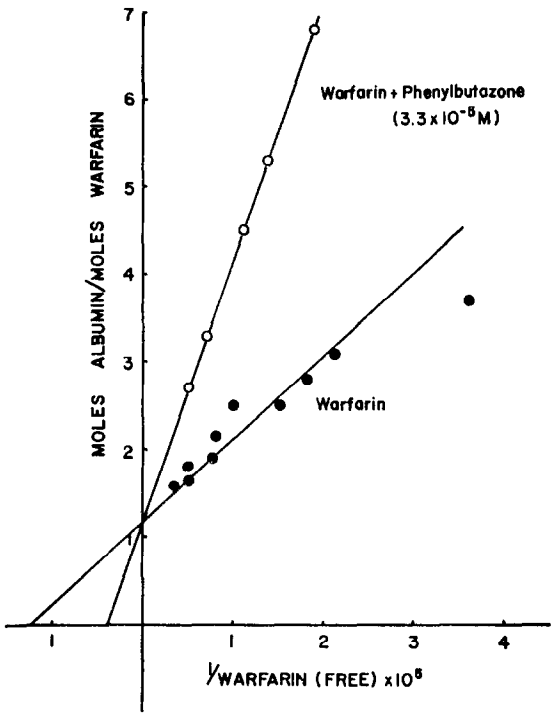

FIG. 2. The effect of phenylbutazone on the binding of warfarin-^{14}C to human albumin. The number in parentheses is the final concentration of phenylbutazone used in all experiments.

97·4 to 62·5 per cent (Table 2). Similarly, as the concentration of CPIB rose from 0 to 270 μg/ml, the binding of warfarin-^{14}C was reduced to 75·1 per cent.

Plasma levels of phenyramidol in human subjects receiving 400 mg t.i.d., by mouth, average 20 μg/ml.[13] When this dose of phenyramidol was employed, there was no decrease in the extent of binding of warfarin-^{14}C.

Protein-bound iodine levels of 10–25 μg/100 ml were observed in human subjects on a maintenance dose of *d*-thyroxine.[14] In the present study, *d*-thyroxine, at a final concentration of 300 μg/100 ml, did not alter the binding of warfarin-^{14}C to albumin.

DISCUSSION

Many drugs are extensively bound to albumin. In man, *bis* hydroxycoumarin is 99 per cent bound and warfarin is 97 per cent bound.[4, 5]

Binding of a drug to albumin can be characterized in terms of the capacity of the protein for the drug (number of sites) and the affinity between the drug and the protein.[15] Recent studies suggest that warfarin binds to a single primary site on

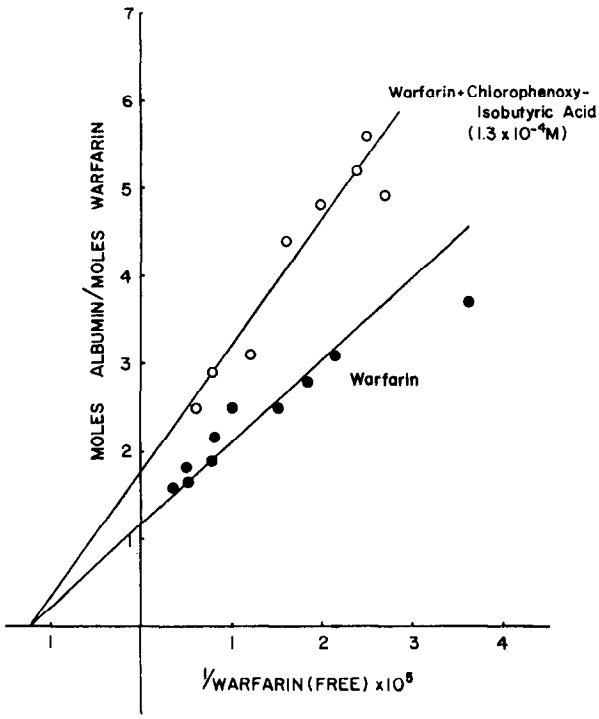

FIG. 3. The effect of chlorophenoxyisobutyric acid on the binding of warfarin-^{14}C to human albumin. The number in parentheses is the final concentration of chlorophenoxyisobutyric acid used in all experiments.

TABLE 2. EFFECT OF "CLINICAL" CONCENTRATIONS OF DRUGS ON THE BINDING OF WARFARIN-^{14}C TO HUMAN ALBUMIN

Drug	(μg/ml)	Warfarin-^{14}C (% bound)
Phenylbutazone	0	97·4 ± 0·5
	50	79·3 ± 1·1
	100	70·6 ± 2·8
	150	62·5 ± 2·3
Chlorophenoxy isobutyric acid	0	97·4 ± 0·5
	90	86·8 ± 2·8
	180	83·6 ± 0·6
	270	75·1 ± 3·9
Phenyramidol	20	96·4 ± 0·7
d-Thyroxine	3	97·0 ± 0·6

In all experiments the final concentration of warfarin was $3{\cdot}5 \times 10^{-2}$ m-mole (11 μg/ml), and the final concentration of albumin was 0·45 m-mole. Results are the mean ± S.D. of 4–6 determinations.

albumin.[16] The binding capacity of normal human plasma for warfarin (mol. wt. = 308) is about 200 μg drug/ml. Since the plasma level of warfarin rarely exceeds 20 μg/ml in clinical practice, the drug in the plasma is almost entirely bound to plasma protein.

Certain acidic drugs interfere with the binding of other drugs to albumin by competing for a limited number of binding sites on the albumin molecule. For example, phenylbutazone reduces the binding of sulfaethylthiadiazole;[3] thiopental displaces secobarbital and pentobarbital from albumin;[17] and salicylic acid increases the concentration of unbound benzoic acid.[18]

In the present study, the effect of various drugs on the binding of warfarin-^{14}C to human albumin was characterized. In certain experiments, an albumin concentration was selected so that the great excess of binding sites for warfarin-^{14}C that occurs *in vivo* was not present. A 0·15 mM, albumin solution binds 94 per cent of warfarin-^{14}C when the final concentration of the labeled compound is $3{\cdot}3 \times 10^{-3}$ m-mole; the theoretical capacity of this albumin solution for warfarin is 46 μg/ml. When the concentration of unlabeled warfarin (62 μg/ml) exceeds the binding capacity of the protein, binding of the labeled drug is considerably reduced (28 per cent bound).

The present study indicates that phenylbutazone competitively inhibits the binding of warfarin to human albumin. Further evidence for a common receptor site on albumin for phenylbutazone and warfarin is suggested by the observation that the K_i for phenylbutazone (165,000) is similar to the affinity constant of phenylbutazone for albumin (125,000).[19]

In other systems, this type of data has been used as evidence of a common transport process for two or more compounds.[20] For example, when two amino acids compete for an identical transport system, the K_i of one of the acids against the other is equal to the affinity constant of that acid for the transport system.[21]

High concentrations of phenylbutazone were less effective than unlabeled warfarin in displacing warfarin-^{14}C from albumin. The affinity of albumin for phenylbutazone may be reduced at high concentrations of the drug.

CPIB is also less effective than unlabeled warfarin in displacing warfarin-^{14}C from albumin. CPIB is a noncompetitive inhibitor of the binding of warfarin-^{14}C to albumin. Although warfarin and CPIB bind to different sites on albumin, the binding of CPIB to the protein may sterically hinder the binding of warfarin, or change the tertiary structure of the protein and thus decrease the affinity between warfarin and its binding site.

d-Thyroxine[22] and sulfaphenazole,[19] compounds that also bind to albumin, are less effective than unlabeled warfarin in displacing warfarin-^{14}C from albumin.

Atropine, an organic base which is bound to numerous primary sites on albumin,[23] does not affect the binding of warfarin-^{14}C to the protein. It thus appears that this basic drug binds to a different site on the albumin molecule.

The pharmacologic activity of certain drugs may be enhanced by other drugs which act to displace them from plasma proteins.[2, 3] Displacement of drug from albumin may result in a lowering of the plasma concentration of drug, since unbound drug is free to distribute in a larger tissue space.[24]

CPIB increases the anticoagulant effect of coumarin-type drugs in man.[7] In the present study, an increase in the unbound fraction of warfarin-^{14}C was produced by CPIB at all concentrations tested. Only modest increases in the unbound fraction of

warfarin-^{14}C are produced by CPIB at concentrations commonly observed during prolonged clinical trials. Consequently, appreciable lowering of plasma level of anticoagulant is not observed in man after treatment with CPIB. Displacement of warfarin from albumin by CPIB probably contributes to the increased anticoagulant effect observed.

Potentiation of the anticoagulant effect of coumarin-type drugs by phenylbutazone has been ascribed to an increase in the unbound fraction of the anticoagulant.[2] Indeed, the present study demonstrates a 10-fold increase in free warfarin when clinical concentrations of the anticoagulant and phenylbutazone are used with a physiological concentration of albumin. However, phenylbutazone also decreases the rate of metabolism of *bis* hydroxycoumarin.[25] Potentiation of the anticoagulant effect of *bis* hydroxycoumarin by phenylbutazone is thus the product of two factors; the extent of their individual contributions is uncertain.

Phenyramidol also increases the anticoagulant response to *bis* hydroxycoumarin.[6] Inhibition of the metabolism of the anticoagulant by phenyramidol has been demonstrated in several animal species. The present study indicates that phenyramidol does not displace warfarin from albumin at clinical concentrations of the drugs. It thus appears likely that the potentiating effect of phenyramidol is due entirely to inhibition of metabolism of coumarin-type anticoagulants.

Studies with *d*-thyroxine in human subjects show that this drug increases the anticoagulant response to *bis* hydroxycoumarin, but that plasma concentrations and half-life of the anticoagulant are unaffected.[7] When concentrations of *d*-thyroxine far in excess of those achieved clinically were used, no significant displacement of warfarin-^{14}C from albumin was observed. The increase in effect of indirect anticoagulants produced by *d*-thyroxine has been attributed to an increase in affinity of the receptor for the anticoagulant.[7]

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