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PLASMA BINDING OF BETAMETHASONE-^{3}H, DEXAMETHASONE-^{3}H, AND CORTISOL-^{14}C— A COMPARATIVE STUDY

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Abstract—The binding of betamethasone (16β-methyl-9α-fluoroprednisolone) and dexamethasone (16α-methyl-9α-fluoroprednisolone) to the proteins of cow, dog, rat and human plasma has been studied *in vitro* by an equilibrium dialysis technique. These synthetic steroids were appreciably bound to the plasma proteins of all species, and the degree of binding did not change substantially over a wide range of concentration. Neither of the synthetic steroids was detectably bound to corticosteroid-binding globulin (CBG) in human plasma, nor did it compete with cortisol for its binding protein.

In human and cow plasma, betamethasone was bound to protein to a lesser extent than was dexamethasone; while this condition held for dog plasma, the difference in binding was not as pronounced. In contrast to the other species, betamethasone was bound to a greater extent than was dexamethasone in the rat.

The synthetic steroids were bound mainly to the albumin fraction of human plasma. Chromatography on Sephadex gel demonstrated that, although albumin has a high capacity for binding dexamethasone, the affinity constant of the steroid–protein complex is of a low order.

THE BINDING of steroids to plasma proteins constitutes an important aspect of their biological activity and has been the subject of several investigations.[1] Indeed, a specific binding protein, corticosteroid-binding globulin (CBG), with a high affinity for cortisol has been demonstrated.[2] Further, it has been shown that CBG-bound cortisol is biologically inactive.[3–5] Much of the accumulated data demonstrate that other steroids also are associated with plasma proteins, and exhibit a wide degree of binding. It has also become increasingly apparent that the binding of the steroids is related to the number and type of substituents on the steroid molecule and that these structural modifications alter the biological potency as well.[6, 7]

Since the bound fraction of the steroid is devoid of biological activity, we have studied the binding of synthetic steroids representing different molecular structures to plasma proteins in order to determine whether the binding of these steroids is related to their activity. The steroids studied were 16α-methyl-9α-fluoroprednisolone (dexamethasone) and its 16β-methyl epimer (betamethasone). Both whole plasma and human plasma albumin binding of the steroids were determined and compared to the results of similar studies with cortisol. Attempts were made, as well, to determine to which of the plasma proteins these steroids were bound and to measure the binding affinity.

MATERIALS AND METHODS

Measurements were facilitated by the use of radiolabeled steroids. Dexamethasone 1,2,4-^{3}H (4·5 mc/mg) was obtained from Belgique A.E.C., betamethasone-16-^{3}H (0·075 mc/mg) from Glaxo Research Ltd., and cortisol-4-^{14}C (0·15 mc/mg) from New England Nuclear Corp. Paper chromatography and countercurrent distribution established the radiochemical purity (> 95 per cent) of these compounds. Human albumin was obtained from Calbiochem.

The equilibrium dialysis technique *in vitro*[8] was used to measure steroid binding, and it was assumed that the material remaining within the dialysis tube upon equilibrium represented steroid bound to protein. Aliquots (1·5 ml) of heparinized human,* cow, dog or rat plasma, or of a human plasma albumin solution were pipetted into sacs of 27/32 cellulose dialysis casing containing a glass rod over which the ends had been folded and secured. The bags were placed into 50-ml glass-stoppered centrifuge tubes containing 10 ml of buffered isotonic NaCl (pH 7·4). Steroid, dissolved in 0·01 to 0·1 ml dimethylformamide, was added to the inner plasma compartment in one preparation and to the outer buffer compartment in the other; besides providing duplicate samples, this procedure made it possible to ascertain that equilibrium had been achieved. The tubes were secured to a vertical turntable enclosed in a constant temperature cabinet maintained at 37° and rotated at 10 rpm for 2 hr. All of the data presented represent the average value of at least two duplicate preparations.

Measurements of radioactivity of plasma and buffer solutions were made in a Packard Tri-Carb liquid scintillation spectrometer. Plasma samples were digested with NCS reagent[9] (Nuclear Chicago Corp.) and counted in a toluene phosphor solution;[10] aqueous samples were counted in a dioxane–naphthalene system.[11] The use of an internal standard made possible the determination of absolute units of radioactivity. Binding was calculated according to the following equation:

$$\% \text{ Bound} = \frac{(\text{Total plasma steroid}) - (\text{Unbound plasma steroid})}{(\text{Total plasma steroid})}$$

in which,

$$(\text{Unbound plasma steroid}) = (\text{Steroid in buffer}) \times \frac{(\text{Plasma water})}{(\text{Buffer water}).}$$

Steroid concentrations at equilibrium were calculated from the observed radioactivity and the initial specific activity of the steroids. The average per cent recovery of radioactivity from each tube was 95·0 ± 0·7 (S.E.M.). Paper chromatography indicated no transformation of either dexamethasone or betamethasone after 2 hr of incubation in human and cow plasma.

To determine to which of the plasma proteins the steroids were bound and the relative affinity of the steroids for the binding protein, plasma was incubated with the radiolabeled steroids at 37° for 75 min, and fractionated by gel filtration at 4° on a 90 × 1·5 cm column of Sephadex G-200 (Pharmacia).[12] Plasma proteins were eluted from the column with 0·1 M Tris-HCl (pH 8·0) in 1 M NaCl at a flow rate of about 2 ml per hr and 1-ml fractions were collected for assay. Protein concentrations were determined by measuring the absorption at 280 mμ in a Beckman spectrophotometer

* Pooled samples of human blood plasma were obtained from the Essex County (New Jersey) Blood Bank.

equipped with a Gilford sample absorbance recorder. Tritium or ^{14}C-radioactivity was then measured in the scintillation counter.

RESULTS

Human plasma

In human plasma, both ^{3}H-betamethasone and ^{3}H-dexamethasone were appreciably bound to plasma proteins over the concentration ranges studied (Table 1). There was a significant difference in the binding of the two components, however. The average binding of betamethasone was 62·5 per cent and that of dexamethasone was 77·4 per cent. Statistical analysis of these data indicated dexamethasone to have significantly greater binding to plasma protein than betamethasone at all concentrations studied ($P < 0{\cdot}01$).

TABLE 1. BINDING OF BETAMETHASONE-^{3}H, DEXAMETHASONE-^{3}H AND CORTISOL-^{14}C IN HUMAN PLASMA

Steroid	Steroid in plasma (μg/ml)		% Bound
	Total	Unbound	
Betamethasone	3·45	1·37	60·4
	0·40	0·14	64·8
	0·04	0·02	60·2
	0·02	0·01	64·6
			62·5 ± 1·3*
Dexamethasone	4·63	1·03	77·8
	0·54	0·11	80·4
	0·06	0·02	75·3
	0·04	0·01	76·0
			77·4 ± 1·1*
Cortisol	3·12	1·28	59·0
	0·42	0·12	72·0
	0·06	0·01	85·1
	0·03	< 0·01	89·0

* Mean ± S.D.

At low plasma concentrations of ^{14}C-cortisol (0·03 μg per ml), 89 per cent of the steroid was bound to the plasma proteins (Table 1). Cortisol binding decreased as its concentration in plasma increased, in contrast to the two synthetic steroids whose binding demonstrated no change over a 100-fold range of concentration.

The binding of ^{14}C-cortisol to human plasma proteins in the presence of unlabeled betamethasone and dexamethasone is shown in Table 2. It is apparent that these compounds, when present in concentrations up to 100 times that of cortisol, did not affect the binding of cortisol to the plasma proteins.

Dog plasma

In dog plasma (Table 3), the average binding of betamethasone was 70·4 per cent at plasma concentrations of 0·04–3·4 μg/ml and that of dexamethasone was 72·7 per cent at concentrations of 0·05–4·4 μg/ml. The greater binding of dexamethasone, however, had statistical significance ($P < 0{\cdot}01$) only at the two higher concentrations

TABLE 2. COMPETITION BETWEEN BETAMETHASONE AND DEXAMETHASONE FOR CORTISOL-^{14}C* BINDING SITES IN HUMAN PLASMA

Steroid	Amt. added (μg)	^{14}C-cortisol (μg/ml plasma)	^{14}C-cortisol (% bound)
Betamethasone	none	0·059	85·8
	0·2	0·060	86·7
	2·0	0·057	85·6
	20·0	0·059	85·5
Dexamethasone	none	0·057	85·8
	0·2	0·060	87·3
	2·0	0·058	86·2
	20·0	0·056	84·8

* Cortisol-^{14}C (0·2 μg) was added in each instance with varying amounts of unlabeled synthetic steroids as indicated.

TABLE 3. BINDING OF BETAMETHASONE-^{3}H AND DEXAMETHASONE-^{3}H IN DOG, COW AND RAT PLASMA

	Dog Steroid in plasma		Cow Steroid in plasma		Rat Steroid in plasma	
Steroid	(μg/ml)	(% bound)	(μg/ml)	(% bound)	(μg/ml)	(% bound)
Betamethasone	3·36	64·7	2·81	52·6	6·16	86·0
	0·39	70·0	0·32	58·7	0·74	88·0
	0·20	72·0	0·03	56·9	0·06	88·0
	0·04	74·7	0·02	57·5	0·03	88·4
		70·4 $\pm$ 2·1*		56·4 $\pm$ 1·3*		87·6 $\pm$ 0·5*
Dexamethasone	4·35	73·5	4·04	72·8	5·66	84·2
	0·49	75·5	0·46	75·3	0·63	86·2
	0·25	73·2	0·05	72·5	0·06	83·0
	0·05	68·7	0·03	74·6	0·03	85·5
		72·7 $\pm$ 1·4*		73·8 $\pm$ 0·7*		84·7 $\pm$ 0·7*

* Mean $\pm$ S.D.

studied. The percentage binding of betamethasone increased as the steroid concentration in plasma decreased and, at the highest concentration, significantly less steroid was bound to protein than at all of the lower levels ($P < 0·01$). In the case of dexamethasone, however, no significant change in binding occurred over a 100-fold range in concentration.

Cow plasma

The results of this study again demonstrated that the synthetic corticoids were bound to plasma protein (Table 3). The average binding of betamethasone was 56·4 per cent over the range of concentrations covered (0·02–2·8 μg/ml plasma) while that of dexamethasone was appreciably higher and averaged 73·8 per cent over its range of concentration (0·03–4·0 μg/ml). As in dog plasma, dexamethasone binding in cow plasma was unaffected by a 100-fold increase in steroid concentration. In contrast to

this, the highest concentration of betamethasone (2·81 μg/ml) was significantly less bound than the lower concentrations.

Rat plasma

The binding of dexamethasone and betamethasone to rat plasma proteins (Table 3) was greater than that observed in any of the species studied. Dexamethasone was 85 per cent bound to plasma protein over a 200-fold range of concentration (0·03–5·7 μg/ml) and betamethasone was 88 per cent bound over a similar range. Statistical analysis of these data indicated that significantly more betamethasone was bound than dexamethasone ($P < 0·01$); in both instances binding was unaffected by changes in steroid concentration.

Steroid binding to albumin and non-albumin proteins

The finding that the degree of dexamethasone and betamethasone binding to human plasma proteins did not change appreciably with changes in steroid concentration, whereas cortisol binding decreased as its concentration increased, suggested that the synthetic steroids were not bound by CBG. That this was indeed the case is indicated by the results of the study of the binding of dexamethasone and betamethasone to 4% human plasma albumin solutions (Table 4). The binding of the steroids in albumin solutions corresponded closely to that observed in plasma; betamethasone was 60 per cent bound and dexamethasone was 84 per cent bound. Steroid binding to albumin was not affected by changes in concentration.

TABLE 4. BINDING OF BETAMETHASONE-^{3}H AND DEXAMETHASONE-^{3}H IN HUMAN PLASMA ALBUMIN SOLUTIONS

Steroid	Steroid in albumin (μg/ml)		
	Total	Unbound	% Bound
Betamethasone	3·15	1·30	58·9
	0·37	0·13	65·1
	0·03	0·01	56·1
	0·02	0·01	59·7
			60·0 ± 1·9*
Dexamethasone	5·45	0·84	84·5
	0·59	0·09	84·9
	0·06	0·01	82·4
	0·03	0·01	83·1
			83·7 ± 0·6*

* Mean ± S.D.

When plasma was dialysed against buffer containing albumin in the same concentration as in the plasma, a slight degree of betamethasone and dexamethasone binding (7–9 per cent) to protein other than albumin was observed (Table 5). No change in binding was observed over a 10-fold range of concentration (0·2–1·8 μg/ml). In contrast, cortisol, at concentrations of 0·3 μg/ml plasma, was bound to non-albumin protein (presumably CBG) 44 per cent, and at a concentration of 0·02 μg/ml (nearer the physiological level) 72 per cent of the cortisol was bound.

TABLE 5. BINDING OF BETAMETHASONE-^{3}H, DEXAMETHASONE-^{3}H AND CORTISOL-^{14}C TO NON-ALBUMIN HUMAN PLASMA PROTEIN*

Steroid	Steroid in plasma (μg/ml)	Non-albumin (% bound)
Betamethasone	1·80	8·4
	0·18	8·5
Dexamethasone	1·82	6·5
	0·19	6·5
Cortisol	1·76	6·3
	0·28	44·0
	0·02	71·5

* Dialysis system contained purified human plasma albumin added to the buffer compartment such that its concentration equalled exactly that of the plasma. Plasma albumin concentration was determined by the method of Reinhold *et al.*[12]

Gel filtration of steroid–protein complex

Gel filtration not only indicated the protein fraction to which cortisol was bound, but also made it possible to differentiate between the affinity of binding of cortisol and dexamethasone to plasma proteins. After 75 min of incubation at 37° in human plasma and gel filtration, ^{14}C-cortisol bound to protein was eluted from the Sephadex column (Fig. 1) and most of the radioactivity was associated with the proteins in the albumin region. It is in this region that the α-globulins of the molecular weight range of CBG are found.[13] In contrast to the pattern observed with ^{14}C-cortisol, ^{3}H-dexamethasone incubated with human plasma and applied to the column was eluted as a single peak unassociated with plasma protein (Fig. 2).

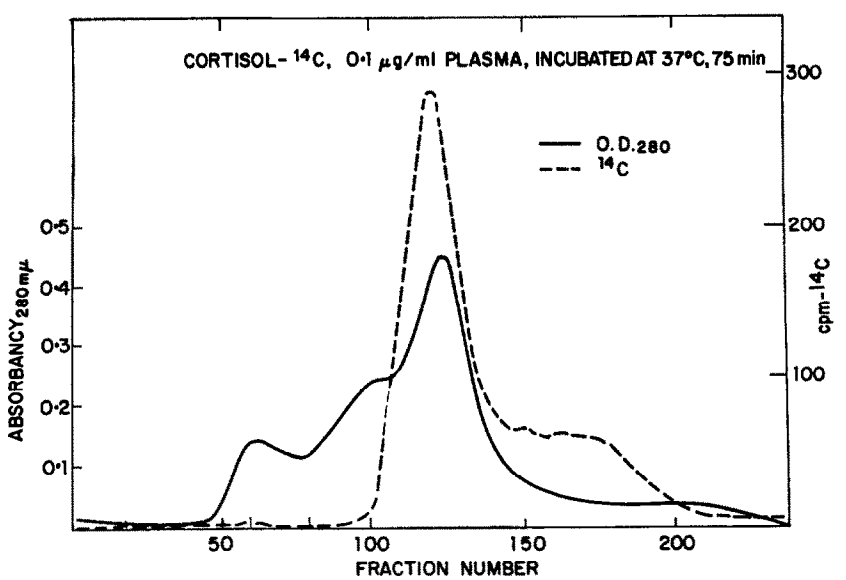

FIG. 1. Elution diagram of cortisol-^{14}C–plasma protein complex from Sephadex G-200.

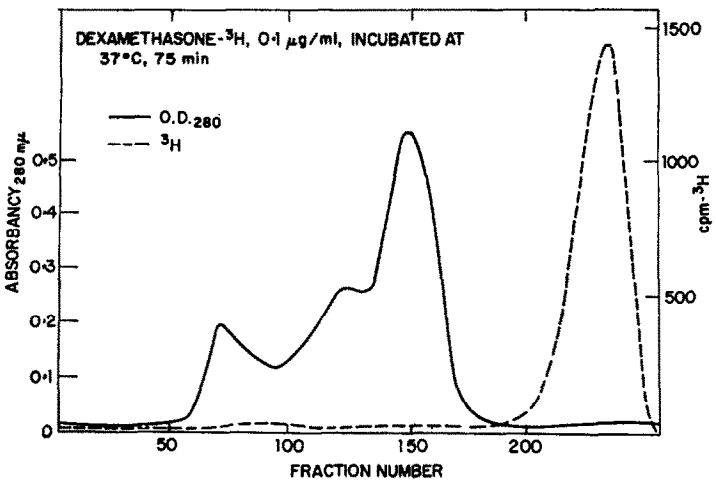

FIG. 2. Elution diagram of dexamethasone-^{3}H–plasma protein complex from Sephadex G-200.

DISCUSSION

The results of this study indicated that betamethasone and dexamethasone were bound to human plasma proteins. The ability of the proteins to bind dexamethasone, however, averaged 22 per cent greater than for betamethasone. Consequently, at approximately equal plasma concentrations of the two steroids, more betamethasone is present in the free state. If one may assume that the unbound steroid is responsible (at least in part) for biological activity, the observation that dexamethasone was bound to a greater degree than betamethasone may partially explain the marginally increased biological activity of betamethasone over dexamethasone in man.[14–19] Protein binding, however, is only one of several factors which may govern biological activity of a steroid; included among these are differences in absorption, excretion, inherent biological activity and metabolism of the steroid.

The study also revealed a striking difference in the capacity of the plasma proteins to bind the synthetic steroids and cortisol. Betamethasone and dexamethasone binding did not change with concentration, whereas that of cortisol decreased appreciably with increasing concentrations. This result suggested that neither synthetic steroid was appreciably bound to CBG, since this protein has a low capacity for binding. Indeed, this finding was further substantiated by the demonstration that dexamethasone and betamethasone were bound mainly to albumin and that only a small portion of these steroids was bound to non-albumin protein. The failure of the synthetic steroids to alter the binding of cortisol indicated that they did not compete with cortisol for its specific protein binding site; these data for betamethasone are consistent with those of a previous report.[20] Furthermore, this lack of competition suggested that the synthetic steroids do not owe their biological effects to the release of bound cortisol.

Gel filtration demonstrated a great difference in the affinity constant(s) of the transcortin–corticoid and albumin–synthetic corticoid binding reaction. It is to be expected that a protein–steroid complex would dissociate continuously while passing through a gel bed devoid of unbound steroid. The CBG–cortisol complex withstood this exhaustive dialysis effect, however, which reflects the very high affinity constant

of this binding reaction. In contrast, the dexamethasone–protein complex underwent complete dissociation during passage through the gel bed in spite of the extremely high capacity of albumin to bind the steroid, thus indicating a very low affinity constant.

In dog and cow plasma, a high degree of binding of the synthetic corticosteroids to proteins was evidenced. Dexamethasone binding in both species was of the same order, while approximately 25 per cent more betamethasone was bound to dog plasma proteins than to those of the cow.

In the cow the binding of dexamethasone to the plasma proteins was 32 per cent greater than that of betamethasone; these data are in substantial agreement with what was observed in human plasma. A further comparison of binding in cow and human plasma indicated that both steroids were less extensively bound to cow plasma proteins.

The results of the study with dog plasma point to a species difference in the binding of the synthetic steroids, since no great difference in the binding of either dexamethasone or betamethasone to the plasma proteins was observed. Additionally, dexamethasone was less extensively bound to dog plasma proteins than to those of human plasma, while the reverse was true for betamethasone.

The highest plasma protein binding of both betamethasone and dexamethasone was observed in the rat and, in contrast to the other species, betamethasone binding was slightly greater than that of dexamethasone. The greater protein binding of betamethasone in the rat may partially explain the increased potency of dexamethasone in this species.[21, 22]

An interspecies comparison of the binding of the two synthetic steroids indicates that the degree and capacity of binding varies in different species. Thus, apart from molecular structure, the binding of steroids is also related to the composition of the plasma. This finding assumes added significance when attempts are made to compare the biological activity of a steroid in different species.

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