Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 227–231. Pergamon Press. Printed in Great Britain

COMPETITIVE BINDING TO MULTIPLE STEREOSPECIFIC BINDING AGENTS AS A MEANS OF VERIFYING THE IDENTITY OF A LIGAND APPLICATION TO CORTISOL IN HUMAN UMBILICAL CORD BLOOD†

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(*Received* 28 *July* 1972)

SUMMARY

Little systematic use has so far been made of comparing apparent relative binding affinities (as indicated by binding competition properties) to multiple stereospecific binding agents as a means of confirming the identity of ligands. That this can provide a relatively simple technique was illustrated for cortisol in umbilical cord blood by assaying the material obtained before and after column chromatography at different concentrations in six different CBG assay systems (dog, cat, horse, monkey, chicken and human). It was also indicated by studies using mixtures of pure steroids. The same principle is equally applicable to the identification of any ligand measured using any type or combination of types of stereospecific binding agent, whether antibody, enzyme, plasma protein or tissue receptor.

No STEREOSPECIFIC binding agent has ever been shown to bind only one ligand. Indeed this would seem highly unlikely. Whether an antibody, enzyme, transin (i.e. naturally-occurring ligand-specific plasma protein), tissue receptor or other agent is used, the specificity of any competitive protein-binding (CPB) assay [1] or other competitive binding assay for a particular ligand in a biological preparation must always be examined very carefully. Although the notion is well accepted that conflicting values obtained using different assay proteins (e.g. different antibodies in radio-immunoassay systems) indicate differences in the entity measured, little systematic use has been made of the reverse concept, i.e. that equal values and similar binding activities (as indicated by similar dilution curves) obtained

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†The following abbreviations and trivial names have been used:

cortisol	F = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione
corticosterone	B = 11β,21-dihydroxy-4-pregnene-3,20-dione
11-desoxycortisol	S = 17α,21-dihydroxy-4-pregnene-3,20-dione
cortisone	E = 17α,21-dihydroxy-4-pregnene-3,11,20-trione
progesterone	P = 4-pregnene-3,20-dione
17α-hydroxyprogesterone	H = 17α-hydroxy-4-pregnene-3,20-dione
11-dehydrocorticosterone	A = 21-hydroxy-4-pregnene-3,11,20-trione
11-desoxycorticosterone	D = 21-hydroxy-4-pregnene-3,20-dione
21-desoxycortisol	DF = 11β,17α-dihydroxy-4-pregnene-3,20-dione
11β-hydroxyprogesterone	HP = 11β-hydroxy-4-pregnene-3,20-dione
pregnenolone	PR = 3β-hydroxy-5-pregnene-3,20-dione.

using a number of assay proteins with different binding properties provide convincing evidence of identity. In applying the ultramicro methods of detection afforded by CPB assays, the problem arises as to the identification of individual ligands in any particular preparation since often only very minute quantities are available for study. One must ask the question "Am I measuring (only) what I think I am measuring?" With only pg to ng amounts to work with, it is difficult to confirm identity by the more usual methods. Merely establishing similarity of binding with that of the standard in a single system by making determinations at different concentrations (i.e. a dilution curve) is insufficient since several ligands may have almost identical affinities for any particular protein. Using two antisera to angiotensin II, Cain *et al.* [2] demonstrated similarity of the dilution curves for valine-5-angiotensin and the reactive material in sheep and accepted this as evidence of identity of sheep angiotensin as the valine-5 form. However might there not be a third form of angiotensin which behaves like the valine form with respect to these two antibodies but which might react differently to a third? How many binding systems are required for positive identification? The present study is intended to consider this question, and to demonstrate the use of multiple binding systems by comparing the predicted and observed results obtained with known mixtures of pure ligands using a number of different binding proteins and by reference to the identification of cortisol in human umbilical cord blood.

The binding proteins used in this study were six species of corticosteroid-binding globulin (CBG, transcortin). These are plasma proteins which bind to a greater or lesser extent the biologically active steroids cortisol, corticosterone and progesterone, their immediate precursors and unconjugated metabolites, and a few other very closely related steroids. The affinity of CBG for each steroid varies from species to species in much the same way as the affinity of various antibodies raised to the same antigen varies for the antigen and its analogues. While the displacement properties of several steroids are similar with respect to any one protein (e.g. D, S, H for chicken CBG; P and HP for monkey CBG; B, H, S, and DF for human CBG – Fig. 1) they differ markedly from one species of CBG to another, so that while two steroids may show identical dilution curves in one system, they cannot be confused if several systems are used. A practical problem which illustrates the use of multiple systems for confirming identity arose in working with the CBG-bound steroids in the human fetus. While in the adult the predominant steroid bound to CBG is normally cortisol, the situation is radically different in the newborn where cortisol forms less than 10% of the total CBG-bound steroid. After column chromatography, assay of the cortisol fraction using several dilutions in the 6 CBG systems provided convincing evidence for identity which was economical both for time and material and did not require a radioisotopic form of the ligand being measured.

MATERIALS AND METHODS

Radioactive tracers with specific activities ranging from 10 to 46 Ci/mmol were purchased from New England Nuclear Corp. On arrival they were diluted to 10 μCi/ml with redistilled ethanol. Non-radioactive steroids were obtained from Sigma Chemical Corp. Pure steroid pools were made up in ethanol to contain the concentrations indicated in Table 1. Suitable aliquots at 2 different concentrations were assayed on three different occasions and the mean and standard error were calculated.

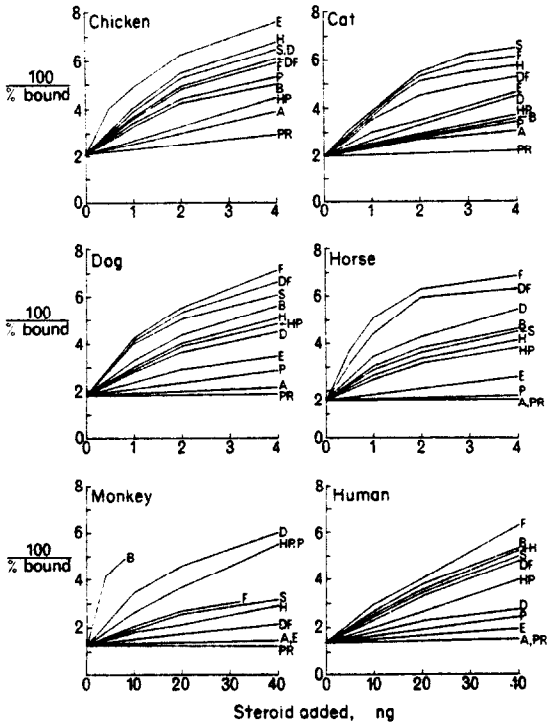

Fig. 1. Standard curves obtained using chicken, dog, horse, cat, monkey and human serum with [^{3}H]-corticosterone as tracer.

Table 1. Observed values* for pure steroid mixtures using 6 different assay systems – ng/ml

Sol'n	Steroid comp'n – ng/ml						Dog	Human	Cat	Chicken	Monkey	Horse
	F	P	E	B	A	S						
1	20	20	20	–	–	–	23·9 ± 1·1	26·9 ± 1·9	30·7 ± 2·6	64·8 ± 7·1	99·3 ± 12·7	20·1 ± 0·5
2	20	10	30	–	–	–	25·9 ± 2·1	25·3 ± 2·3	27·6 ± 2·2	74·6 ± 11·6	54·3 ± 3·8	2·8 ± 2·9
3	20	30	10	–	–	–	21·8 ± 0·7	27·3 ± 1·0	26·0 ± 1·5	60·5 ± 5·5	112·0 ± 7·8	19·7 ± 0·9
4	10	30	20	–	–	–	16·8 ± 1·3	17·7 ± 0·7	21·3 ± 2·0	61·5 ± 10·0	126·3 ± 13·9	11·2 ± 0·5
5	60	–	–	10	–	–	56·9 ± 3·4	69·2 ± 3·9	58·5 ± 2·2	77·7 ± 8·4	160·0 ± 16·5	56·1 ± 3·1
6	60	–	–	–	10	–	51·6 ± 3·8	59·7 ± 2·2	53·8 ± 2·6	64·0 ± 9·6	56·6 ± 2·7	55·3 ± 3·5
7	60	–	10	–	–	–	54·9 ± 1·9	64·1 ± 3·7	61·2 ± 3·1	88·8 ± 7·0	65·3 ± 5·4	60·0 ± 3·2
8	60	–	–	–	–	10	57·9 ± 1·6	71·9 ± 4·6	64·8 ± 5·1	85·6 ± 13·1	69·4 ± 7·7	60·0 ± 3·1

*Mean ± S.D.

One ml of umbilical cord serum from each of 20 infants was pooled, added to 30,000 c.p.m. of F (about 100 pg) and extracted twice with 5 volumes of ethyl acetate. The combined extracts were passed through a column of Sephadex LH-20 as described previously [3, 5] using methylene chloride: methanol (98 : 2 v/v), a solvent system observed to separate F from all other known competitors for CBG binding-sites. Two ml eluate fractions were collected. An aliquot (usually 0·02 ml) of each eluate was counted, and those from the radioactive area were combined. Appropriate aliquots at several different concentrations were evaporated to dryness and assayed in duplicate in six separate experiments using each CBG system. Aliquots of the original pool were assayed for "total corticoids" in terms of cortisol after extraction only.

The assay systems used were those described previously [4] using dog, cat, chicken, monkey and human CBG. In addition, horse serum was used in similar fashion. [^{3}H]-Corticosterone (4·0 μCi/100 ml) was used as the tracer throughout. Florisil was used as the adsorbent for all systems (40 mg, except for human CBG where 100 mg was used). The reciprocal of the % bound × 100 was calculated for each sample, the values for the standards were plotted against the ng standard added, and the steroid in each sample taken as the mean of values measured at various dilutions, was calculated accordingly. The standard curves for the various steroids studied are shown in Fig. 1.

RESULTS

The results in Table 1 show the values for pure steroid mixtures. The presence of large amounts of competitors other than F (solutions 1–4) was readily detected by highly significant differences ($P < 0{\cdot}01$) among the values in the six systems. Even when the competing steroid was reduced to only 1/7 of the total steroid content (solutions 5–8) it was detected ($P < 0{\cdot}05$) except for A which is a weak competitor in all six systems. Where values among at least 4 systems were in agreement, (i.e. $P > 0{\cdot}05$) as for solutions 2, 6, 7 and 8, all but one of the values were within ± 3 S.D. of the true value.

Assays of the F fraction after a single chromatography on Sephadex LH-20 are compared with each other and with those obtained for crude extracts in Table 2. The widely differing values for crude extracts clearly indicate non-homogeneity

Table 2. Steroids measured as cortisol in umbilical cord serum

CBG	Observed steroid concentration in terms of cortisol – ng/ml*	
	Before chromatography	after chromatography†
Dog	144 ± 5	69 ± 4
Cat	195 ± 18	81 ± 5
Horse	118 ± 4	76 ± 3
Chicken	1266 ± 87	71 ± 11
Monkey	1700 ± 144	95 ± 11
Human	173 ± 9	72 ± 5

*Mean ± S.D. (n = 6).
†Corrected for recovery of 78%.

of the binding activity. Of the CBG's used, horse CBG appears to be the most specific for cortisol since it gives the lowest value, while chicken and monkey CBG are the least specific. From Fig. 1 it is clear that monkey CBG is highly specific for corticosterone rather than cortisol. Values for the chromatographed fraction of cortisol do not differ significantly from each other in the six systems, indicating that the material behaves as cortisol when compared with authentic cortisol with respect to its binding to 6 different proteins.

Although not indicated individually here, the various dilutions used in determining mean values in both experiments gave results consistent with the expected error of the method, i.e. the dilution curves for each sample in each system were compatible with those of the standard. The use of multiple dilutions minimizes the possibility of artefactitious values due to nonspecific interference (i.e. blanks).

DISCUSSION

The values for cord blood obtained in this study are in good agreement with those of others using double isotope dilution methods, i.e. 7·8 μg/100 ml [6] and 9·2 μg/100 ml [7]. Lack of consistency of the values for the crude plasma extract clearly indicated the need for purification; had the values been similar, purification would have been unnecessary. Consistency of low results in all 6 systems after purification indicated that only one binding substance was present and that this behaved as cortisol. When disparate values are found for different binding systems, the binding protein giving the lowest value for a particular preparation (in this case horse) is presumably the one most specific for the standard ligand against which measurement is being made. While horse CBG is specific enough for routine clinical cortisol estimation [8], it cannot be expected to be adequately specific for cortisol in unfractionated umbilical cord serum where the P concentration may be as much as 100-fold greater, and where cortisone levels exceed those of cortisol.

From the data presented for pure mixtures and for steroids in plasma, it is concluded that consistency of results obtained by CPB assay using several different proteins with high affinity for a particular ligand provides convincing evidence for the identity of the material being measured as that of the standard. While it is unlikely but possible that 2 ligands may react very similarly in two binding systems (e.g. F and S in the monkey and dog systems) it is highly unlikely that they will react similarly in three and negligibly likely in four.

ACKNOWLEDGEMENT

The technical assistance of Mme Renée Haidar and Miss Doreen Lobo is gratefully acknowledged.

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