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STUDY OF STEROID-PROTEIN BINDING BY A NOVEL "TWO-TIER" COLUMN EMPLOYING CIBACRON BLUE F3G-A-SEPHAROSE 4B. I—SEX HORMONE BINDING GLOBULIN

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SUMMARY

Cibracron Blue F3G-A-Sepharose gel was utilized as an affinity matrix for the removal of albumin from plasma prior to gel filtration on Sephadex LH-20. The incorporation of affinity chromatography and gel filtration in the same column allows for direct and simultaneous evaluation of steroid binding to both non-specific and specific binding components in human plasma. The binding parameters of Sex Hormone Binding Globulin, SHBG, are presented.

The application of 5α-Dihydrotestosterone-3(*O*-carboxymethyl) oxime–human serum albumin, DHT-3-CMO–HSA, conjugate used in conjunction with Cibacron Blue F3G-A-Sepharose is discussed together with results on the relative affinity of steroid derivatives pertinent to the affinity purification of SHBG. The usefulness of this affinity matrix in enrichment of steroid haptens and its possible application to purifying antibodies is also discussed.

INTRODUCTION

The use of conventional gel filtration in studies of steroid binding to specific proteins such as Corticosteroid Binding Globulin, CBG [1, 2], and SHBG [3] relies on the dissociation of the non-specific steroid–protein complex and therefore on a certain column size. That this dissociation is complete has already been shown [3]. However, there is some dissociation of the stronger specific complexes as well on the relatively large columns that have so far been in use; this may give less than maximal values for Binding Capacity, BC, of the specific protein. The dissociation of the specific complex is dependent on sample size, column size, the time the sample remains in the column, and the temperature. Up till now the dissociation of the specific complex could only be minimized by using low temperatures and/or large sample volumes. Burke's Steady-State gel filtration [4] has the disadvantage of requiring very large sample volumes to generate Steady-State conditions and is time consuming. None of the above techniques offer direct information on the non-specifically bound steroid.

We have utilized the dye-linked Sepharose, which was shown to remove human serum albumin [5], as the upper, affinity section of our "two-tier" column (Fig. 1); this is stacked on top of a lower gel filtration column consisting of Sephadex LH-20. The high capacity, low affinity steroid–albumin complex is effectively removed in the upper (affinity) part of the column. Due to the nature of this adsorption dissociation of the steroid–albumin complex is negligible in this part of the column under the conditions employed. Due to the retention of a significant portion of the steroid in the affinity compartment the size of the gel filtration column and therefore the size of the sample can be reduced considerably allowing for rapid passage of the specific complex and thereby minimizing its dissociation. Direct information on the albumin bound, real free steroid, and specific protein bound steroid can be obtained by counting the radioactivity in the affinity gel, the Sephadex LH-20 and the eluate respectively, in the scintillation system we have described.

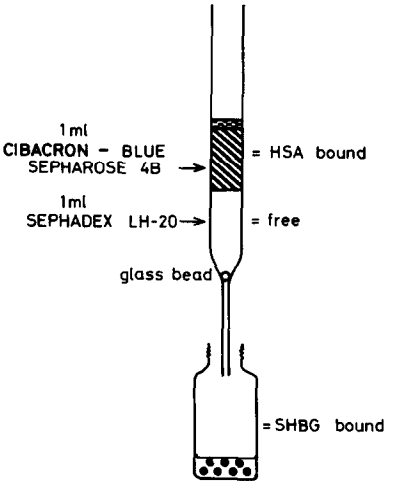

Fig. 1. Diagram of "two-tier" column.

Steroid nomenclature:
17β-hydroxy-5α-androstan-3-one = 5α-Dihydrotestosterone (DHT); 3β, 17β-Dihydroxy-5α-androstane = Androstanediol; 1,3,5,(10)-Estratriene-3,17β-diol = Estradiol

BC measurements using our method were carried out on groups of normal subjects, women pill users, pregnant subjects, hirsute women, and men with cancer of the prostate.

Competition studies involving steroid derivatives were carried out and the DHT-3-CMO HSA conjugate was investigated as an affinity adsorbant with a view to future purification of SHBG. It was found that only the conjugate with a low steroid:HSA ratio is adsorbed on to the dye-linked Sepharose gel, this has important bearing on the enrichment of steroid haptens for the purpose of raising antisera [6].

MATERIALS AND METHODS

The Cibacron Blue F3G-A dye was a gift from Dr R. L. Stonely, Ciba-Geigy, U.K. Dyestuffs and Chemicals Division. Sepharose 4B and Sephadex LH-20 were purchased from Pharmacia (G.B.) Ltd. 5α-Dihydrotestosterone (DHT), crystallized and lyophilized chicken, human, rabbit, and bovine serum albumins were obtained from Sigma Chemical Co. (St. Louis, U.S.A.) DHT was recrystallised before use. The DHT-3-(*O*-carboxymethyl) oxime, DHT-3-CMO, (m.p. 161°) was synthesised from 1 g DHT containing 5 μCi tritiated DHT and coupled to human serum albumin, HSA, using the mixed anhydride procedure previously described [7]. The molar ratio of steroid: albumin was 7.3:1 as determined by the radioactivity in the conjugate. Androstanediol-3-hemisuccinate (m.p. 215°; m.p. for epiandrosterone-3-hemisuccinate was 252°) and estradiol-3-hemisuccinate (m.p. 192–194°) were prepared as described [8, 9]. Tritiated DHT (1,2,4,5,6,7,H^3) specific activity 100 Ci/mM, was purchased from the Radiochemical Centre, Amersham, Bucks., U.K. All other reagents and solvents were of analytical grade. The buffer employed was 0.05 M Tris, 0.005 M $CaCl_2$/HCl, pH 7.3. Radioactivity in column eluate fractions and exuded 1 ml vols of Cibacron Blue F3G-A-Sepharose and Sephadex LH-20 gels was counted in a Wallac 20,000 liquid scintillation counter using a 3:2 Toluene·Triton X100 scintillant containing 3 g/l PPO. Plasma samples from heparinised cubital vein bloods were stored at −20° until used.

Preparation of Cibacron Blue F3G-A-Sepharose. The Cibacron Blue F3G-A-Sepharose was prepared by the method of Travis *et al* [5]:- 200 ml of washed Sepharose 4B were mixed gently with an equal vol. of 1 N NaOH containing 1 g of $NaBH_4$. 4 ml of epichlorohydrin was added and the mixture maintained at 60° for 1 h. The gel was then washed until the washings were neutral, and 200 ml of the gel mixed with an equal vol. of water, heated to 60° and 200 mg of the Cibacron Blue F3G-A dye in 20 ml of water added slowly and the mixture stirred for 15 min. 20 g of NaCl were then added after which the mixture was heated to 80° and 4 g of Na_2CO_3 added. After a further 30 min the Cibacron Blue Sepharose was washed with hot water until no traces

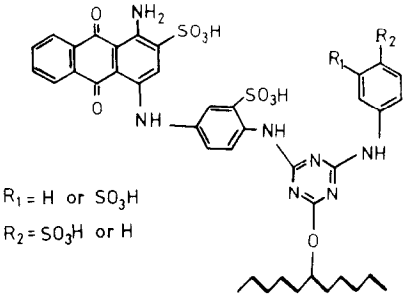

Fig. 2. Structure of Cibacron Blue-F3GA ligand.

of dye were detected by measuring the absorption at 260 nm. The gel was then finally washed with Tris buffer. The ligand concentration was determined by subtraction of the dye concentration in the washings. The structure of the gel is shown in Fig. 2.

Determination of the albumin-adsorbing capacity of Cibacron Blue–Sepharose. Columns of 1, 2, and 3 ml of the Cibacron Blue–Sepharose were set up in Pasteur pipettes (10 × 0.7 cm), washed once with Tris buffer and 0.5 ml of a 5% HSA solution applied. Eluate fractions of 2× column vol were collected and the E_{280} determined. Similarly the binding of bovine serum albumin, chicken serum albumin and rabbit serum albumin was investigated. Under the same conditions 100 μl samples of a 5% solution of HSA preincubated with 40,000 d.p.m. of tritiated DHT and 10 ng radio-inert DHT for 1 h at 4° were applied to a 1 ml column and the radioactivity in 6 × 1 ml fractions of the eluate and in the gel determined. At the same time the amount of steroid bound to HSA was determined by the charcoal separation method previously described [10].

Use of Sephadex LH-20 to free steroid. Columns as above were set up containing 1, 2, 3, and 4 ml of Sephadex LH-20 swollen in Tris buffer. To these 40,000 d.p.m. of tritiated DHT and 20 ng of radio-inert DHT were added (final S.A. = 2,000 d.p.m./ng). Six washes of the column were collected and the radioactivity determined in these and the exuded Sephadex LH-20 gels. Under the same conditions a parallel experiment was run employing columns of the Cibacron Blue–Sepharose gel.

Optimising of column conditions to minimise dissociation of SHBG–steroid complex. Effect of temperature and column size: Pregnancy plasma diluted 1:20 was incubated with tritiated DHT (*circa* 150,000 d.p.m./ml) for 45 min at 4°. 200 μl of this incubate was applied in duplicate to columns of LH-20 (1, 2, 3, and 5 ml) set up in pipettes as above and maintained at room temperature in one case and under 8° in the other. The radioactivity in the void vols and that remaining in the gel of these columns was determined (Fig. 4).

Effect of sample size and column size: Aliquots of neat pregnancy plasma and dilutions of 1:5, 1:10, 1:15, and 1:20 were applied incubated with tritiated DHT as above. 200 μl of the incubates were applied in duplicate to 1 ml and 2 ml of LH-20 maintained

at 8° with ice-cold Tris buffer (Fig. 5). In parallel experiments the radioactivity bound for all the incubates was determined using the charcoal separation method.

For the 1:5 and 1:10 dilutions the experiment was repeated at room (21°) and under 8° using 100 μl samples and applying them to "two-tier" columns (Fig. 1), consisting of a lower 1 ml Sephadex LH-20 layer and an upper layer of 1 ml Cibacron Blue–Sepharose. Radioactivity was determined in the eluates (void vols) and the glass columns were cut at the interfaces of the two gels which were then exuded and counted separately. The charcoal method was used again for the determination of binding at 4° in 100 μl of the incubates. The same procedure was applied to heat-treated plasma.

Scatchard analysis of SHBG using "two-tier" columns. Two-tier columns, as above were prepared and the DHT binding to SHBG in plasma samples determined in the following manner: All the samples for Scatchard analysis [11] were diluted 1:10 in Tris buffer; 0.4 ml aliquots of pregnancy plasma were incubated with 0, 1, 2, 5, 10, 15, 25, and 50 ng of DHT. Samples from non-pregnant women and men were incubated 0, 0.5, 1, 2, 4, 7, 14 and 25 ng DHT and 0, 0.5, 1, 2, 4, 7 ng DHT respectively. Human milk samples were diluted 1:5 and incubated with 0, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 ng DHT. All incubates had 120,000 d.p.m. of tritiated DHT added and the incubation time was 45 min at 4°. 100 μl of the incubates were applied in duplicate to the "two-tier" columns and the radioactivity in the eluted buffer (3 ml) and that in the gel layers determined. Scatchard plots were then constructed to assess the binding constants. In addition Scatchard plots were constructed for a male plasma sample using the above method and using the charcoal separation method on the plasma before and after albumin depletion in the following manner:— 0.5 ml of neat plasma was applied to a 7 ml column of the Cibacron Blue-Sepharose gel and eluted with 5 ml of Tris buffer (dilution = 1:10).

Rapid assessment of binding capacity of clinical samples using the "two-tier" column system. 0.4 ml Aliquots, diluted 1:5 of pregnancy, female and male plasma were incubated with 40, 25, 10 ng of DHT respectively and 120,000 d.p.m. of tritiated DHT. For hirsute women the BC measurements were carried out using 10 ng of DHT and 25 ng. Similarly, men with cancer of the prostate had BC determinations at 10 and 25 ng levels of DHT carried out. As above, 100 μl of the incubates were applied in duplicate to the two-tier columns and 3 ml eluates and the gel layers counted.

Competition studies involving steroid derivatives. Late pregnancy plasma was diluted 1:10 and 0.4 ml aliquots of it were incubated with (a) 0, 2, 5, 10, and 25 ng DHT; (b) 12.5, 25, 250 ng and 5 and 10 μg of DHT-3-CMO; (c) 12.5, 25, 250 ng and 5 and 10 μg of androstanediol-3-hemisuccinate; and (d) 12.5, 25, 250 ng and 5 and 10 μg of estradiol-3-hemisuccinate. All incubates had 120,000 d.p.m. of tritiated DHT. The two-tier column system was employed to assess the binding in the usual manner. The ability of these steroid derivatives to displace tritiated DHT was investigated by constructing competing standard curves for these steroid derivatives. Cross-reactions were expressed according to the equation:

$$\frac{\text{mass of DHT required to displace 50\% tritiated DHT}}{\text{mass of } X \text{ required to displace 50\% tritiated DHT}} \times 100 .$$

where X is the steroid derivative.

Affinity immobilisation of SHBG. Neat, late pregnancy plasma, 0.5 ml, was dealbuminized as described, the 5 ml eluate collected was then made up to 15 ml in Tris buffer. 7 ml of fresh Cibacron Blue–Sepharose gel was stirred with 4 ml of a solution of DHT-3-CMO–HSA conjugate (13.6 mg HSA/ml) containing 537 d.p.m. per mg, for 55 min at room temperature. The liquid was drained on a column (4.5 × 1.1 cm) and the affinity matrix washed with 30 ml of Tris buffer until the optical density and the d.p.m. in the washes were nil.

2 ml Aliquots of the diluted plasma sample were incubated with 1, 2, and 3 ml of the conjugate linked affinity gel for 15 min at room temperature and 30 min at 4°, the suspensions were shaken on a Vortex mixer every five min while at room temperature and every ten mins while at 4°. Similarly, 2 ml aliquots of the same plasma sample were incubated with corresponding volumes of Cibacron Blue–Sepharose not previously incubated with the DHT-3-CMO–HSA conjugate. At the end of the incubation the gels were drained on Pasteur pipette columns and 0.4 ml of the drained buffer aliquots were incubated in duplicate in assay tubes with 100,000 d.p.m. tritiated DHT for 45 min at 4° prior to separation of the bound from free by the charcoal method. The remaining drained buffer from the conjugate linked Cibacron Blue–Sepharose columns was scintillated and radioactivity in it determined to see if there was any leakage of the DHT-3-CMO–HSA conjugate from the columns. Radioactivity in the conjugate linked affinity gels was determined separately.

RESULTS

The procedure employed for coupling the dye, Cibacron Blue F3G-A, to Sepharose 4B resulted in ligand concentrations of Cibacron Blue F3G-A of 0.3–0.4 μmol per ml of Sepharose 4B. Batches of the Cibacron Blue–Sepharose showed consistently adsorbing capacities for HSA of around 5 mg per ml of gel (4.8, 5.8, 5.6, 5.4 mg per ml of gel for four batches separately). Determination of the HSA adsorbing capacity using 1, 2, and 3 ml gel vols in Pasteur pipettes gave results of 4.3, 4.5, and 4.8 mg HSA bound per ml of gel respectively, for the first batch of Cibacron Blue–Sepharose. Experiments using samples of bovine, chicken, and rabbit serum

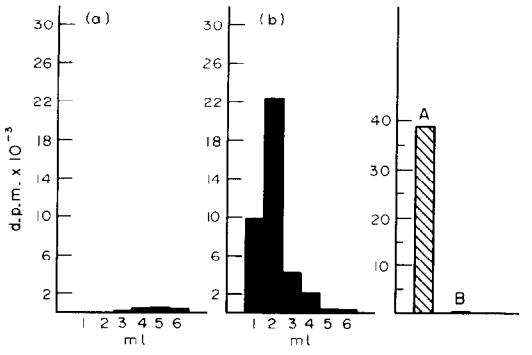

Fig. 3. Histograms of retention of steroid by (A) 1 ml SepharoseLH-20,(B) 1 ml in Cibacron Blue–Sepharose. Black areas indicate radioactivity in 1 ml fractions of the eluate, hatched areas indicate radioactivity retained by the gels.

albumins resulted in no evidence of binding. Determinations of DHT binding to HSA on the Cibacron Blue–Sepharose column and those obtained by using the charcoal technique gave figures of 65.2 and 63.7% respectively.

Investigation into the suitability of Sephadex LH-20 for entrapment of free steroid showed that a 1 ml column of Sephadex LH-20 was adequate to retain the free steroid. The results obtained using the 1 ml Sephadex LH-20 column are illustrated in Fig. 3. The figure also shows that 1 ml of Cibacron Blue–Sepharose does not retain free steroid. On the basis of these results the "two-tier" column system of 1 ml Cibacron Blue–Sepharose and 1 ml Sephadex LH-20 was designed to fractionate the albumin and steroid concentrations in the ensuing assay methodology. Investigations into the effects of temperature and column size (Fig. 4) showed that minimum dissociation of the total bound DHT in plasma diluted 1:20 occurred at a temperature under 8° using a Sephadex

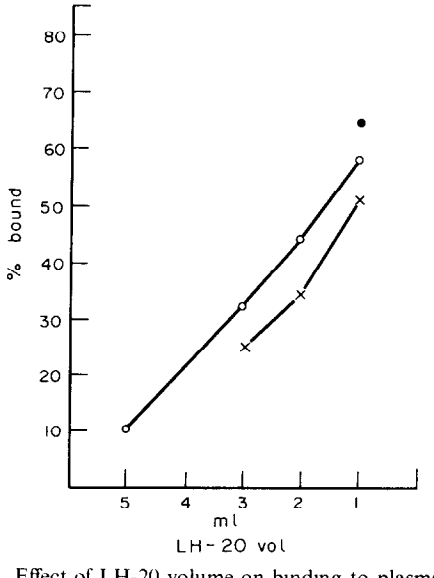

Fig 4. Effect of LH-20 volume on binding to plasma protein, ie. per cent [^{3}H]-DHT in void vols (O——O = below 8°, ×——× = room temperature, O = per cent bound determined by charcoal method at 4°)

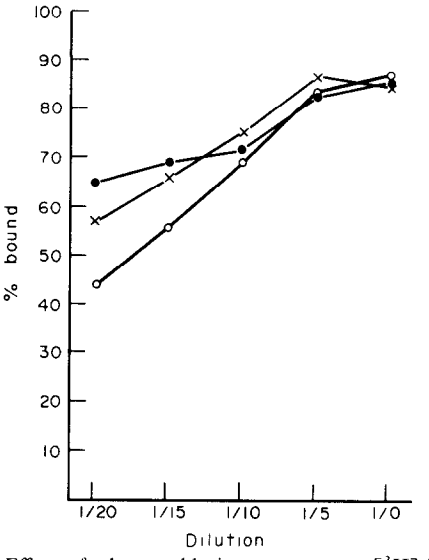

Fig. 5. Effect of plasma dilution on percent [^{3}H]-DHT bound to plasma protein using, 1 ml Sephadex LH-20 (×——×), 2 ml Sephadex LH-20 (O——O) and charcoal (O——O) for separation of bound and free. Column temperatures = below 8

column volume of 1 ml. Sample sizes that can be safely employed using 1 ml of Sephadex LH-20 at under 8° are 100 µl of a 1:5 or 1:10 dilution (Fig. 5). At room temperature however, a 1:5 dilution is required to prevent dissociation of the SHBG and albumin bound DHT on the column (Table 1).

Scatchard analysis of a male (Figs 6A, B), female and late pregnancy plasma gave BC values for DHT

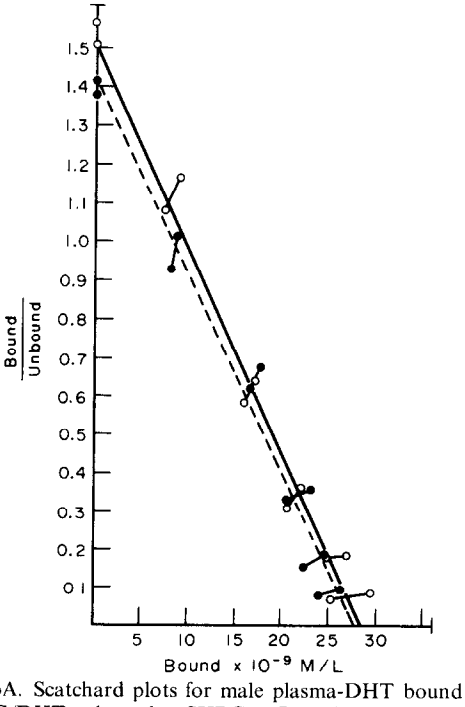

Fig 6A. Scatchard plots for male plasma-DHT bound to SHBG/DHT unbound to SHBG vs DHT bound to SHBG Solid line and open circles = charcoal method applied using de-albuminised plasma. Dotted line and full circles = two-tier column method applied to untreated plasma

Table 1. Percentage binding in eluates and blue gel compartments of "two-tier" column system (TTC) compared for dilutions of 1:5 and 1:10 at two temperatures and related to binding obtained by charcoal method at 4° for untreated and heat-treated plasma

Temp C	Plasma dilutions	Untreated plasma: Total % binding assessed by charcoal method at 4	Untreated plasma: % binding assessed by TTC: Eluate (SHBG bound)	Untreated plasma: % binding assessed by TTC: Binding in blue gel (Albumin bound)	Heat-treated plasma: % binding assessed by charcoal method at 4° (a)	Heat-treated plasma: % binding in eluate of TTC (residual SHBG) (b)	(a b)
21	1 5	90 6	64 72 (total = 94 02)	29 3	34 5	3 4	31 1
	1 10	71 4	40 6 (total = 62 9)	22 3	23 1	0 6	22 5
below 8°	1 5	90 6	63 4 (total = 94 7)	31 2	35 5	5 0	30 5
	1 10	71 4	44 8 (total = 72 4)	27 6	25 4	0 7	24.7

of 2.7, 6.5 and 30.1 × 10^{-8} M/L respectively. Human milk gave a BC value of 1.38 × 10^{-9} M/L. The association constant, K_a, values obtained for DHT were 0.55, 0.74, and 0.87 × 10^8 L/M for male, female and late pregnancy plasma samples respectively, at 8°. Human milk had a K_a value for DHT of 0.175 × 10^8 L/M at 8° (Fig. 7).

Scatchard plots obtained using the two-tier column system compare well with those obtained using the charcoal method (Figs. 6A, B). In Fig. 6A Scatchard plot from the two-tier column system for a male plasma sample is compared with the plot obtained for the same sample using the charcoal method on dealbuminized plasma. Similarly, taking the total bound (i.e. albumin bound as determined by counting the Cibacron Blue–Sepharose, and SHBG bound as determined from the eluates) as the bound, B, in Scatchard analysis we get a plot similar to that obtained when the charcoal method is applied to the undenatured sample of the same plasma (Fig. 6B).

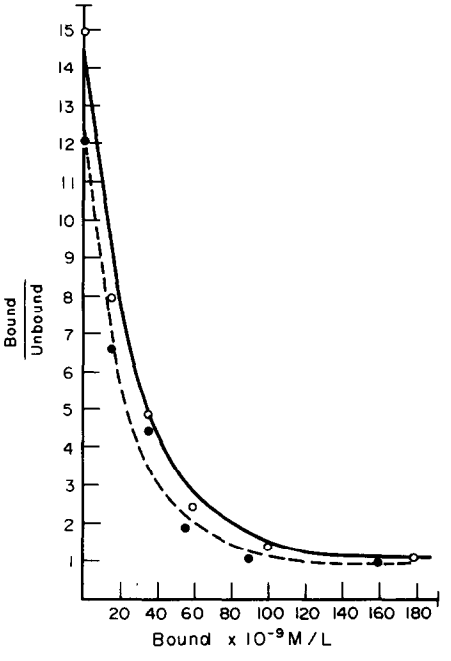

Fig. 6B. Scatchard plots for untreated male plasma. "Bound" = DHT bound to HSA and SHBG, obtained using the charcoal method (solid line and open circles) and by the two-tier column method through adding blue gel (albumin) bound to eluate (SHBG) bound.

BC values obtained for groups of clinical samples are listed in Table 2A and 2C. The intergroup variation is highly significant (Table 2B). In addition BC values in plasma samples from three hypogonadal males were determined; these were all in the male range, 1.16, 0.68, and 0.82 μg DHT bound per 100 ml plasma, though the group was not large enough for statistical analysis. The inter-assay variation was determined by replicate analyses of a male plasma pool (20 samples). Mean and standard deviation for ten determinations in duplicate was 0.828 ± 0.0223 μg DHT bound/100 ml plasma (range 0.80–0.87) and the coefficient of variation was 2.69%.

Competition studies using DHT-3-CMO, androstanediol-3-hemisuccinate, and estradiol-3-hemisuccinate showed only minor percent cross-reactions with DHT, 0.6, 0.25 and 16% respectively.

As previously reported [6] the binding of the DHT-3-CMO–HSA conjugate to Cibacron Blue–Sepharose was lower than that for unconjugated HSA, 3.07 and 3.46 mg/ml; the latter figure was obtained after incubation of the gel with DHT-3-CMO–HSA for 55 min, the former by application of the conjugate sample to a 3 ml column of the gel set up in Pasteur pipette. The material that was adsorbed on the Cibacron Blue–Sepharose was of a lower steroid:HSA ratio, 3:1, (2.5:1 previously

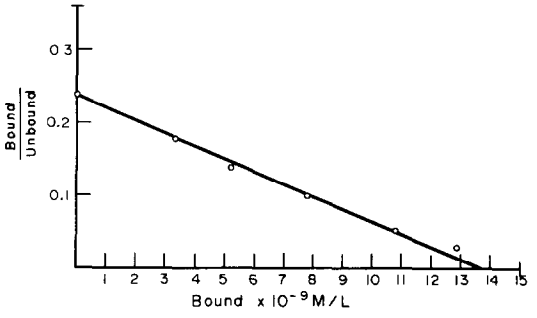

Fig. 7. Scatchard plot for a human milk sample obtained using the two-tier column method. DHT bound to SHBG/DHT unbound to SHBG vs DHT bound to SHBG is plotted.

Table 2A. Binding capacity values obtained using "two-tier" column method, expressed as μg DHT bound/100 ml plasma

Group A normal females	Group B normal males	Group C female pill users	Group D hirsute females	Group I Ca prostate males
1.56 (70)	0.84 (770)	3.6 (60)	1.28 (20)	2.73 (15)
2.05 (75)	1.17 (1030)	2.57 (40)	0.89 (60)	1.39 (30)
1.5 (40)	0.81 (625)	2.73 (25)	0.88 (110)	4.5 (40)
1.93 (35)	0.86 (620)	2.41 (85)	1.35 (55)	3.21 (70)
1.58 (45)	0.82 (365)	2.78 (25)	1.21 (75)	1.86 (75)
1.82 (56)	0.70 (395)	2.81 (30)	1.27 (45)	8.78 (65)
1.96 (60)	0.54 (440)		1.07 (45)	1.71 (10)
1.60 (65)	0.75 (525)		0.88 (140)	10.61 (70)
	0.63 (435)		0.64 (45)	6.74 (10)
	0.64 (555)		0.61 (150)	
	0.58		1.15 (25)	
	0.71		0.97 (100)	
	0.86		0.88 (100)	
$n = 8$	$= 13$	$= 6$	$= 13$	
mean = 1.745	= 0.76	= 2.817	= 1.0	
SD = 0.209	= 0.1684	= 0.412	= 0.238	
range = 1.5–2.05	= 0.58–1.17	= 2.57–3.6	= 0.6–1.28	

Figures in brackets = plasma testosterone concentrations in ng/100 ml

Table 2B Significance between groups A, B, C, D and G from Table 2A, C determined by Student's t test

Group	A	B	C	D	G
A		<0.001	<0.001	<0.001	0.001
B	<0.001		<0.001	<0.01	0.01
C	<0.001	<0.001		<0.001	0.001
D	<0.001	<0.01	<0.001		0.001
G	0.001	0.01	0.001	0.001	

Table 2C Binding capacity values for pregnancy samples and cord plasma samples expressed as μg DHT bound/100 ml plasma. Figures in brackets = weeks of pregnancy

Group F pregnancy samples	Group G cord plasma
6.23 (24)	0.49
4.4 (20)	0.35
6.6 (26)	0.49
6.8 (27)	0.56
7.24 (32)	0.39
12.09 (34)	
10.58 (32)	$n = 5$
13.87 (37)	mean = 0.456
9.67 (33)	SD = 0.0842
14.24 (36)	range = 0.35–0.56
8.44 (34)	
10.93 (36)	
13.4 (37)	

reported [6]) and was only marginally successful in removing SHBG The 1, 2, and 3 ml columns removed 41, 56, and 64% of SHBG binding activity from 2 ml of a 1:30 dilution of dealbuminized late pregnancy plasma

DISCUSSION

Many previous attempts to determine the BC of SHBG have emphasized the need to circumnavigate the non-specific binding of albumin for steroids. There is now available the means not only of adsorbing albumin specifically from plasma samples but also of determining the compartmentation of steroids between the non-specific and specific binding components of human plasma.

The two-tier column system described here enables the BC to be determined in a single step, a procedure involving 20 min or so. For the purpose of a simplified assay the temperature of the column is maintained below 8° by application of ice cold buffer. This step could be improved upon by, for example, performing the assay in a cold room. In general, the method allows for rapid determination of BC of clinical samples (one technician can handle up to 20 samples at any one time) and the results we obtained using various clinical groups showed significant differences in BC for DHT whereas the accepted routine measurements of total plasma testosterone and DHT have shown no differences between, for example, groups of hirsute and normal women. The BC values reported in this work are generally in good agreement with those reported elsewhere [12–15].

Our investigations have shown that use of the mixed anhydride procedure for coupling DHT-3-CMO to HSA is not very successful in making an affinity ligand suitable for adsorption on Cibacron Blue–Sepharose. Only material with a low steroid:protein ratio is retained by Cibacron Blue–Sepharose due perhaps to some structural changes brought about in the HSA molecule by the coupling procedure and/or due to the involvement of available lysine residues. Furthermore, the steroid derivative, DHT-3-CMO, employed in the immobilisation of SHBG had a very low affinity for SHBG and consequently the DHT-3-CMO–HSA was not effective as a means of removing SHBG for purification purposes. The fact that only the conjugate of a low steroid:protein molar ratio (apparently < 3:1) is bound to Cibacron Blue–Sepharose gel is fortuitous and interesting in itself as reported [6] in that the steroid:HSA conjugates required for eliciting antibodies for radioimmunoassay purposes can be enriched by removing this low steroid:protein ratio material. Therefore, for the purposes of large scale purification of SHBG further development of the mode of coupling the steroid ligand to HSA, so as to retain the ability of HSA to adsorb on to Cibacron Blue

Sepharose, is needed. It may be possible to achieve this by utilisation of available glutamic acid residues on the HSA and coupling the hapten through a bifunctional bridge such as hexane–diamine which may permit better HSA-dye interaction. Improved immobilisation of SHBG may be possible by using a steroid derivative which has a greater affinity for SHBG such as estradiol-3-hemisuccinate.

This approach to studying steroid–protein binding may find application in receptor studies. Often in the study of receptor binding of endogenous steroids the interference due to specific binding proteins in the plasma has to be evaluated. For example, the SHBG contaminant in receptor studies could be removed if a suitably specific steroid–HSA conjugate could be employed for the affinity adsorption of this plasma protein provided that the receptor or the intracellular protein was investigated for binding to another steroid, e.g., it has been reported that in cancer of the prostate there is binding of testosterone to a CBG–protein, CBG having been shown not to bind DHT [16]. Thus it is feasible that a DHT–HSA conjugate might be employed to remove the contaminating SHBG and the specific testosterone–CBG interaction studied. Conversely, the binding of testosterone or estradiol to SHBG could be studied in the plasma without interference from CBG (CBG binds to both these steroids) if CBG could first be removed by employing, say, a cortisol–HSA conjugate for its adsorption.

Since steroid–bovine serum albumin conjugates are widely used for producing antisera for specifically determining steroid concentrations in radioimmunoassay systems, cross-reacting species of antibodies might be removed by employing analogous HSA derivatives of the cross-reacting steroid species and immobilising such by passage through a Cibacron Blue–Sepharose column.

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