A solid-phase radioimmunoassay for human corticosteroid binding globulin

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ABSTRACT

A radioimmunoassay (RIA) for human corticosteroid binding globulin (CBG) has been developed using $^{125}$I-labelled CBG and a monospecific solid-phase CBG-antiserum (CBG-Ab-cellulose). In an RIA of serum CBG concentrations, pure CBG standards (1–100 ng protein) or samples (1:200) were incubated (16 h at 20°C) with $^{125}$I-labelled CBG and CBG-Ab-cellulose. After addition of 2 ml 0.9% NaCl, the tubes were centrifuged, supernatants were aspirated and the $^{125}$I-labelled CBG bound to the CBG-Ab-cellulose pellet was counted. The specificity of the RIA was confirmed by parallel displacement curves for serial dilutions of male, female and pregnancy sera, as well as pure CBG standards. The mean ± s.d. recovery (99 ± 8%) of pure CBG (1–6–25·0 ng) added to a diluted serum sample verified the accuracy of the method, and a good correlation ($r = 0·97; n = 43$) existed between serum CBG cortisol binding capacity (nmol/l) measurements and CBG concentrations (mg protein/l) measured by RIA. Intra- and interassay precisions (C.V.) at low to high serum CBG concentrations were <5% and <9%, respectively. The mean ± s.d. serum CBG concentrations (mg protein/l) measured by the RIA were: 21·8 ± 4·6 in boys ($n = 12$), 20·0 ± 4·2 in girls ($n = 9$), 20·7 ± 2·7 in men ($n = 6$), 20·5 ± 2·9 in women ($n = 6$) and 47·1 ± 10·5 in pregnant women ($n = 5$). The sensitivity of the standard curve used in the routine RIA of serum CBG was 1·0 ng CBG/assay tube, but this could be increased to 0·2 ng/assay tube by reducing the amount of CBG-Ab-cellulose used. The RIA is suitable for both clinical and research purposes, and will aid the identification of abnormal forms of CBG and facilitate studies of the regulation of CBG production in vitro.


INTRODUCTION

Corticosteroid binding globulin (CBG) is the major corticosteroid transport protein in human plasma and until recently measurements of the protein have been based on its steroid binding capacity, which may be determined by a variety of methods including: equilibrium dialysis (Daughaday, Adler, Mariz & Rasinski, 1962), ultrafiltration (Kaene, Pearson & Walter, 1969), steady-state polyacrylamide gel electrophoresis (Hansson, Purvis, Attiramadal et al. 1977) and a DEAE ion-exchange filter disc assay (Schiller & Petra, 1976). Steroid binding capacity measurements, however, rely on the assumptions that the characteristics of steroid binding proteins under investigation are identical within a given species, and that non-specific interactions between tracer ligand and other proteins are negligible and/or constant in all samples. The steroid binding activity of CBG is also highly temperature and pH dependent, and the presence of steroids or drugs may interfere competitively with measurements of binding capacity. In addition, the sensitivity of steroid binding assays are limited by the specific activity of $^3$H-labelled steroid tracers, and the number of samples which may be processed simultaneously is often limited by the complexity of assay protocols.

The development of methods for the isolation of pure CBG (Rosner & Bradlow, 1971; Le Gaillard, Racadot, Racadot-Leroy & Dartreuves, 1974) has enabled several laboratories to produce specific anti-
CBG antisera which have been used to detect the presence of CBG by immunodiffusion (Rosner, Darmstadt & Toppel, 1973), and to measure concentrations of the protein by radial immunodiffusion (Van Baelen & De Moor, 1974) and rocket immunoelectrophoresis (Racodot, Racodot-Leroy, Le Gaillard & Dautrevaux, 1976). More recently, radioimmunoassay (RIA) methods have been developed which require relatively small amounts of specific anti-CBG antiserum, and employ either polyethylene glycol (PEG) or second antibody precipitation as a means of separating CBG-antibody-bound complexes (Bernutz, Häsle, Horn et al. 1979; Rosner, Polimeni & Khan, 1983). Although widely used, these separation techniques are characterized by several limitations; PEG when used alone or in conjunction with a second antibody may yield erroneous results in serum samples from lipaemic subjects (Desbuquois & Auerbach, 1971; Wickus, Morden, Thoftne & Davis, 1982), while the use of a second antibody requires an additional incubation step for optimal results. We have therefore developed a simple, solid-phase RIA for CBG that overcomes these problems and is ideal for routine or research use.

MATERIALS AND METHODS

Chemicals

Cortisol and corticosterone were obtained from Sigma (London) Chemical Co. Ltd, Poole, Dorset, and were used without further purification. [1,2-3H]Cortisol (sp. act. 47 Ci/mmol) and Bolton and Hunter reagent were obtained from Amersham International plc, Bucks. When necessary, [3H]cortisol was purified on 1 ml Lipidex-5000 (Packard-Becker B.V., Chemical Operations, Groningen, The Netherlands) chromatography columns, using hexane:chloroform (60:40, v/v) as elution solvent. Sepharose CL-4B, DEAE-Sepharose CL-6B, blue Sepharose CL-6B and Sephadel G-100 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrogel AcA44 and Isogel Agarose-EF were from LKB-Produktur AB, Bromma, Sweden. Polyampholytes (Servalyt AG 3-5) were from Serva Feinbiochemica GmbH & Co., Heidelberg, F.R.G. Picrylsulphonic acid (sodium salt) and ethylene-diamine were from Aldrich Chemical Co. Ltd, Gillingham, Dorset. Cellulose (Sigmacell type 20), dextran (av. mol. wt 70 000), carbodiimazole, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and 1,4-butanediol diglycol ether were from Sigma. Molecular weight standards and reagents for polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate (SDS)-PAGE, as well as standard laboratory reagents and solvents, were of analytical reagent grade and were purchased from BDH Chemicals Ltd, Poole, Dorset. The assay buffer was 0.14 M-phosphate buffered saline, pH 7.4, containing 0.1% gelatin and 0.1% sodium azide (PBS) and the dextrancoated charcoal (DCC) solution was PBS containing 0.25% charcoal (Norit A. Amend Drug and Chemical Co., Irvington, NJ, U.S.A.) and 0.025% dextran. The sheep anti-rabbit immunoglobulin G antiserum (SAR) was a gift from Dr P. Lähteenmäki, University of Helsinki, Finland.

The affinity ligand, 11β-hydroxy-androst-4-en-3-oxo-17β-carboxylic acid (HACA), was prepared according to Le Gaillard et al. (1974), and the product was shown to be pure by silica-gel thin-layer chromatography (chloroform: ethanol: water, 80:19:1, by vol.). Diaminoethylxirane-Sepharose CL-4B was prepared as described by Sundberg & Porath (1974), and the coupling of HACA (1 mg/ml gel) was catalysed by the addition of 10 mg EDAC/ml gel in 70% 1,4-dioxan, at pH 4.5. Non-covalently bound steroid was then removed by extensive washing with 80% methanol. This produced an affinity gel with a binding capacity of 3.2 nmol CBG/ml, and which could be regenerated and used repeatedly (Mickelson, Harding, Forsthoevel & Westphal, 1982).

Purification of CBG

The purification procedure was performed at 4°C unless otherwise stated. Human pregnancy serum (0·2 l) was dialysed for 16 h against 10 mm-Tris–HCl buffer, pH 8·5, and centrifuged (25 000 g) for 30 min. The supernatant was applied to a DEAE-Sepharose CL-6B column (25 × 5 cm) and eluted with a linear 0·02-0·40 M-KCl gradient. The eluate fractions were screened by a cortisol binding capacity assay (see below), and fractions containing CBG were applied to a HACA–diaminoethylxirane-Sepharose CL-4B column (10 × 2·6 cm) at 10 ml/h. After washing the column with 10 mm-Tris–HCl, pH 7·5, 0·2 M-KCl, 10% dimethylformamide (TKD), CBG was displaced from the column by incubation (25°C for 30 min) with 1 column volume of TKD buffer containing 5 mg cortisol/50 ml, and eluted with TKD buffer. The affinity purified CBG was concentrated, desalted and washed with 30 ml 10 mm-Tris–HCl, pH 7·5, in an Amicon Ultrafiltration cell using a PM 10 filter (Amicon Ltd, Bucks). The concentrate (5 ml) was then applied to a blue Sepharose CL-6B column (6 × 1·6 cm) to remove albumin, while CBG eluted in the drop-through. Final purification was achieved by preparative PAGE (Bethesda Research Laboratories Inc., Maryland, U.S.A.) on a 10 × 1 cm polycrylamide rod gel (total % of acrylamide monomer (T) = 10%, amount of cross-linking agent as a % of total acrylamide (C) = 2.5%) containing 1 mm-[3H]cortisol and 2 μm-cortisol. The CBG was eluted from the gel with the electrophoresis buffer (0·1 m-borate buffer, pH 8·5), and fractions con-
containing CBG-bound [3H]cortisol were concentrated by ultrafiltration through a PM 10 filter. Approximately 2 mg pure CBG were obtained, representing a 25% yield. The pure protein exhibited a specific activity of 23 400 pmol cortisol binding capacity/mg protein, which represents approximately a 1600-fold purification.

Gel electrophoresis

The purity of CBG at each stage of the purification was assessed by PAGE, using a modification of the method of Davis (1964). Samples were analysed on horizontal polyacrylamide gels (T = 7.5%, C = 2.5%, 250 × 114 × 3 mm) using 0.1 M-glycine–Tris (GT) buffer, pH 8.9 (10 °C), as electrophoresis buffer. Electrophoresis was performed at 400 V (constant voltage) using bromphenol blue (BPB) as tracking dye, and proteins in the gels were identified by staining with Coomassie brilliant blue R250. Pure CBG and 125I-labelled CBG (see below) were also subjected to electrophoresis (200 V constant voltage) in a 1% agarose gel using 0.2 M-GT buffer, pH 8.9. The position of the BPB marker was noted upon completion of the electrophoresis, and lanes containing 125I-labelled CBG were cut into 2 mm segments and counted in an LKB Multi-gamma counter (LKB/Wallac Oy, Turku, Finland). Pure protein was fixed with 1.2% picric acid in 17% acetic acid and identified by staining with Coomassie brilliant blue R250 (Fig. 1).

The molecular weight of CBG was determined under denaturing conditions by SDS–PAGE using 50 mm-phosphate buffer, pH 7.0, containing 0.2% (w/v) SDS, as electrophoresis buffer. Coomassie brilliant blue R250 was used to identify the proteins, and the electrophoretic mobilities (Rp) of CBG and the molecular weight standards (cytochrome c, myoglobin, chymotrypsinogen A, ovalbumin, bovine serum albumin and ovotransferrin) were determined relative to BPB at four different (T = 5.0, 7.5, 10.0 and 12.5%) acrylamide concentrations (C = 2.5%). Some glycoproteins display anomalous behaviour with respect to their mobility on SDS–PAGE gels (Breit张家, 1971; Banker & Cotman, 1972), but a Ferguson plot (Ferguson, 1964) revealed that the relative electrophoretic mobilities of CBG and the standards versus %T, intersect at ~0%T. This indicated that they all possess equivalent charge densities, and a molecular weight of 59 500 was determined for CBG by comparing its retardation coefficient with those of the standards.

Gel filtration on Ultrogel AcA44

The molecular weight (63 800), Stokes radius (35.2 Å) and frictional ratio (1.36) of CBG were determined by gel filtration (Siegel & Monty, 1966) on an Ultrogel AcA44 chromatography column (85 × 1.6 cm) using ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin and aldolase as protein standards, and establishing the void and column volumes with blue dextran 2000 and potassium dichromate respectively.

Isoelectrofocussing

Analytical isoelectrofocussing (IEF) of pure CBG was performed on thin layer horizontal agarose (1%) gels (0.5 × 250 × 114 mm) containing 2% polyampholytes, pH 3–5. The gels were focussed for 2 h at 6 W constant power; protein was then fixed with 11.4% trichloroacetic acid and 3.4% sulphosalicylic acid, and stained with Coomassie brilliant blue R250. This revealed six major bands between pH 3.8 and 4.2.

Amino acid and carbohydrate composition

The results of the amino acid analysis are the means of ten separate determinations. The CBG was either oxidized with performic acid to stabilize half-cystine and methionine and hydrolysed in 6 M-HCl (n = 4), or hydrolysed in 4 M-methanesulphonic acid and 3(2-aminoethylindole) to stabilize tryptophan (n = 6). The amino acid composition determined in terms of number of residues/mol protein (calculated using a molecular weight of 59 500 and a carbohydrate content of 23%) were as follows: aspartic acid 43, threonine 29,
serine 41, glutamic acid 43, proline 10, glycine 35, alanine 26, half-cystine 8, valine 25, methionine 17, isoleucine 15, leucine 50, tyrosine 10, phenylalanine 18, lysine 16, histidine 14, arginine 12 and tryptophan 5. These data were also used to calculate the partial specific volume of CBG (0.73 ml/g), as described by Lee & Timasheff (1974).

The qualitative and quantitative composition of the monosaccharide components of pure CBG were determined after acid hydrolysis and trimethylsilyl-ether derivitization, by gas–liquid chromatography (Krusius, Finne, Kärkkäinen & Järnefelt, 1974). The carbohydrate content (23%) was determined with respect to the protein concentration measured by the Bradford (1976) assay. The relative amounts (mol/mol CBG) of the various carbohydrate moieties identified were as follows: fucose 0.8, mannose 16.2, galactose 11.6, N-acetylglucosamine 20.4 and sialic acid 13.3.

**Preparation of 125I-labelled CBG**

Pure CBG was radiiodinated using the Bolton and Hunter reagent, as recommended by Amersham International plc. The 125I-labelled CBG was separated from small molecular weight 125I-labelled complexes on a Sephadex G-100 column (20 × 1 cm), which was pre-equilibrated and eluted with 50 mm-phosphate buffer, pH 7.5, containing 0.25% gelatin as carrier protein. Approximately 14–20% of the 125I-label was bound to the protein, and specific activities of 8–12 µCi/µg protein were obtained. When analysed by agarose gel electrophoresis 125I-labelled CBG exhibited one peak of radioactivity, corresponding to the Rf value of CBG (Fig. 1). Pure 125I-labelled CBG was stored at −20°C in 200 µl aliquots containing 2% horse serum, and little evidence of radiolysis or decline in immunological activity was observed after 2 months of storage.

**Preparation and characterization of antiserum**

Anti-human CBG antiserum was produced in a rabbit according to the method of Vaitukaitis, Robbins, Nieschlag & Ross (1971). The animal was given an initial multiple site, subcutaneous and intramuscular immunization (50 µg CBG in Freund's complete adjuvant), followed by several booster immunizations (50 µg CBG in Freund's incomplete adjuvant) over a 4-month period. Ten days after the final booster, exsanguination was achieved by cardiac puncture, and the blood was allowed to clot overnight at 4°C. Antiserum was then prepared by centrifugation at 3000 g for 30 min.

The presence of antibodies against CBG and the approximate equivalence point of precipitation between antibody and antigen were determined by double immunodiffusion in 1% agarose gels. The monospecificity of the antiserum was demonstrated by the immunoelectrophoretic method of Grabar & Williams (1953) and tandem crossed-immunoelectrophoresis, according to Clarke & Freeman (1968), against purified CBG and human pregnancy serum (Fig. 2).

**Preparation of CBG antibody-cellulose (CBG-Ab-cellulose)**

Antibodies against CBG were covalently linked to carbodiimidazole-activated, microparticulate cellulose by incubating (16 h at 20°C) a 1:5 dilution of antiserum (in 50 mm-barbitone buffer, pH 8.0) with activated cellulose (5:1, v/w), as described by Chapman & Ratcliffe (1982). A titre curve was prepared by
incubating 5–100 µl CBG-Ab-cellulose (20 mg/ml PBS buffer) adjusted to 100 µl with unreacted cellulose (20 mg/ml PBS buffer), together with 100 µl 125I-labelled CBG for 16 h at 25°C. After the addition of 2 ml 0.9% NaCl, the tubes were centrifuged (2000 g for 5 min), the supernatants aspirated and the cellulose pellets counted. A 1:5 dilution of stock CBG-Ab-cellulose resulted in 62% of maximum binding and was chosen for serum CBG measurements, while a 1:20 dilution which gave 25% of maximum binding was used to measure CBG at low concentrations.

Standards

The concentration of the pure CBG standard was defined by both cortisol binding capacity assay (see below), and the Bradford (1976) protein assay using human serum albumin as the standard. Serial dilutions of pure CBG standard were prepared in PBS containing 2% horse serum to prevent loss of pure protein at very low concentrations. Horse serum was used for this purpose as it does not cross-react with the anti-human CBG antiserum (Robinson, Hawkey & Hammond, 1985). When the cortisol binding capacity of the CBG standards was determined, an excellent correlation ($r = 0.99$; gradient $42 \text{ mg protein/µmol binding capacity}$, intercept 0) was found between CBG protein concentration and cortisol binding capacity, after the cortisol binding capacity (2 nmol/l) of the 2% horse serum had been subtracted (Fig. 3).

CBG binding capacity assay

The cortisol binding capacity of CBG was determined by a saturation analysis method (Hammond & Lähteenmäki, 1983) in which samples were prediluted 1:100 in a DCC solution, and incubated at 20°C for 30 min to remove endogenous steroids. After centrifugation (3000 g for 10 min), the supernatants were diluted in PBS as appropriate (1:5 for serum measurements) and aliquots (100 µl) were incubated (1 h at 20°C) with 10 nM [3H]cortisol in the presence and absence of 2 µM-cortisol. After a further incubation at 0°C (15 min), non-CBG-bound steroids were removed by incubation (10 min at 0°C) with 500 µl DCC solution and centrifugation (3000 g for 10 min). The supernatants were added to 2.5 ml Rialuma (Lumac B.V., Basle, Switzerland) and counted for 10 min, or until 10,000 counts had accumulated. The cortisol binding capacity (nmol/l) was calculated from the amount of [3H]cortisol bound specifically to CBG, after correction for recovery and sample dilution.

RESULTS

Specificity

In addition to the demonstration (by standard immunoelectrophoretic techniques) that the rabbit anti-CBG antiserum was monospecific when tested against human pregnancy serum, the CBG-Ab-cellulose would also immunoprecipitate CBG from serum: for example, the cortisol binding capacity of 500 µl diluted (1:100) pregnancy serum (700 nM CBG/l) decreased by 55% when incubated together with 500 µl CBG-Ab-cellulose for 16 h at 20°C.

Assay specificity was also demonstrated by parallelism between serial dilutions of various human serum samples and the pure CBG standards (Fig. 4). This indicated that the CBG was immunologically identical in all samples, and that cross-reacting or interfering substances were not present in different human sera. The mean ± S.D. concentrations of CBG in serum samples ($n = 5$) were also measured by RIA before (21.2 ± 1.4 mg/l) and after (21.0 ± 1.6 mg/l) treatment

Radioimmunoassay

Duplicate tubes containing 100 µl CBG standard or a 1:200 dilution of serum samples in PBS + 2% horse serum, 100 µl 125I-labelled CBG (40,000 d.p.m.) and 100 µl CBG-Ab-cellulose (2 mg cellulose) were vortex mixed and left to stand for 16 h at 20°C. Physiological saline (2 ml) was added to all tubes, which were then centrifuged (5 min at 2000 g). The supernatants were aspirated to waste, and the tubes containing the cellulose pellet were counted in an LKB/Wallac Multigamma counter for 2 min, or until 10,000 counts had accumulated. Calculation of results was accomplished using the RIA spline curve fitting program supplied by LKB/Wallac.
CBG/assay using for The sample CBG/100 A

(30 min at 20°C) with a DCC solution to remove endogenous steroids (Hammond & Lähteenmäki, 1983) and clearly showed that the RIA measurement was independent of the presence of steroid at the CBG binding sites, and that treatment with DCC did not result in any loss of CBG.

Accuracy
A series of pure CBG standards (1-6-250 ng CBG/100 µl) were added to an equal volume of a serum sample (1:100 dilution) with a very low CBG content, as measured by cortisol binding capacity (160 nmol/l) and RIA (8-6 mg/l). The concentrations of CBG were then measured to determine the recovery of CBG from the serum and this was 99 ± 8% (n = 5).

Sensitivity
The ranges and sensitivities of dose-response curves for pure CBG standards were determined using different amounts of CBG-Ab-cellulose (Fig. 5). The sensitivity of the standard curve (x B₀ = 2 s.d.) obtained using a 1:20 dilution of CBG-Ab-cellulose was 0-2 ng CBG/assay tube, and this corresponded to an approximate 20000-fold dilution of pregnancy serum. In contrast, the sensitivity of the standard curve obtained using a 1:5 dilution of CBG-Ab-cellulose was 1-0 ng CBG/assay tube, and a 50% displacement of the 125I-labelled CBG occurred at 10 ng CBG/assay tube, which corresponded to a 1:200 dilution of serum from men and non-pregnant women.

Precision
Repeated measurements (n = 6) of control sera containing low (14-1 ± 0-52), medium (35-1 ± 0-72) and high (57-8 ± 1-08) concentrations of CBG (mean ± s.d. mg/l) were performed to assess the intra-assay variability, and when expressed as coefficients of variation were 3-7, 2-1 and 1-9% respectively. Interassay (n = 5) coefficients of variation for the same samples were 4-3, 8-2 and 8-1% respectively.

Serum measurements
When measured by RIA the mean serum CBG concentrations (mg protein/l), and ranges in prepubertal boys and girls were similar to those in adult men and women, but were generally increased in women taking oral contraceptives and were 100-200% higher in women during late pregnancy (Table 1). The mean cortisol binding capacities (nmol/l) and ranges of the same samples are also presented in Table, and the relationship between serum CBG measurements by RIA and the cortisol binding capacity assay are shown in Fig. 6. A good correlation (r = 0.97) existed between
TABLE 1. Serum concentrations of corticosteroid binding globulin (CBG): the concentration of CBG was measured in serum samples by radioimmunoassay (RIA) and by a cortisol binding capacity assay.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>RIA (mg/l)</th>
<th>Binding capacity (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boys</td>
<td>12</td>
<td>21.8 (18.3–28.3)</td>
<td>490 (288–623)</td>
</tr>
<tr>
<td>Girls</td>
<td>9</td>
<td>20.0 (14.3–26.7)</td>
<td>482 (346–736)</td>
</tr>
<tr>
<td>Men</td>
<td>6</td>
<td>20.7 (18.8–25.2)</td>
<td>424 (348–572)</td>
</tr>
<tr>
<td>Women</td>
<td>6</td>
<td>20.5 (14.9–22.9)</td>
<td>445 (375–513)</td>
</tr>
<tr>
<td>Women (on O.C.†)</td>
<td>5</td>
<td>31.5 (20.2–52.3)</td>
<td>666 (414–1078)</td>
</tr>
<tr>
<td>Women (late pregnancy)</td>
<td>5</td>
<td>47.1 (31.5–60.0)</td>
<td>1063 (708–1419)</td>
</tr>
</tbody>
</table>

*mg protein as measured by the Bradford assay.
†O.C. = oral contraceptives.

The values obtained by the two methods, and the gradient of the regression line indicated that 1 mg (CBG by protein) was equivalent to 22.3 nmol cortisol binding capacity, which was very similar to the specific activity of the standards (Fig. 3). An exception to this close relationship was found and is shown by the open circle in Fig. 6. This late pregnancy serum sample exhibited approximately half the cortisol binding capacity expected with respect to CBG concentration measured by RIA, and repeated analyses have shown that assay errors do not account for this observation.

**DISCUSSION**

The use of affinity chromatography was introduced by Rosner & Bradlow (1971) for the purification of CBG and in our experience it gave a 410-fold purification of the protein which, when analysed by PAGE and SDS–PAGE, revealed only one major contaminant, i.e. serum albumin. This was removed by affinity chromatography using a group specific absorbant (Cibacron blue F3G-A) which binds albumin with high affinity. The CBG obtained at this stage appeared to be pure by electrophoretic analyses and was used to immunize a rabbit, but analysis of the resultant anti-serum by immunoelectrophoresis (Grabar & Williams, 1953), using pregnancy serum as the antigen, revealed the presence of a second antibody in addition to that directed against CBG. The relative mobility of the contaminating antigen was similar to that of immunoglobulin and preparative electrophoresis was therefore introduced to remove this contaminant. This gave a homogeneous preparation of CBG which produced high titres of a monospecific antiserum.

The molecular weight of pure CBG was determined under native and denaturing conditions and estimates of 63 800 and 59 500 were obtained respectively. In addition, when serum was subjected to gel filtration and the RIA was used to quantify CBG in the eluates, only one peak of immunoreactive CBG was identified with an identical molecular weight to that of the pure protein (data not shown). This indicates that the molecular weight and conformation of CBG does not change during purification, and that CBG exists as a monomer in serum, unlike pure CBG which gradually forms dimers (Mickelson et al. 1982) or polymers (dimers and trimers) upon storage (Rosner, 1972; Robinson, 1984). The amino acid composition of pure CBG is similar to that reported by others (Fernlund & Laurell, 1981; Bernutz et al. 1979; Mickelson et al. 1982), and its carbohydrate content is almost identical to that reported by Strelchyonok, Avvakumov, Matveentseva et al. (1982) and Mickelson et al. (1982).

We originally used the chloramine T method to radioiodinate CBG, but found the product was unstable when stored at −20 °C, probably as a result of chemical damage to the protein or radiolysis due to the high specific activity. We therefore used the Bolton and Hunter reagent, and although the specific activity of the resulting tracer was lower (8–12 µCi/µg protein) than that obtained by the chloramine T method (68 µCi/µg), it was stable for at least 2 months and gave background counts of <3% of the total activity used in the RIA.

Previously a combination of binding capacity measurements and molecular weight determinations have been used to estimate CBG protein concentrations but these parameters vary considerably depending on the methods used. We therefore used the Bradford protein assay to determine the protein content of our pure CBG standards because it is a convenient method of protein estimation and should yield com-
parable results in all laboratories. Although some proteins behave anomalously when measured in this way, Macart & Gerbaut (1982) recently reported that inclusion of SDS in the reagent equalizes the method to all proteins, and when this was done no differences in the measurements of CBG concentrations were observed. In addition to recovery studies, evidence for the accuracy of the present method is provided by the relationship between the protein concentration and cortisol binding capacity of CBG in serum samples, especially as this indicates that 44 843 g protein is equivalent to 1 mol CBG if one steroid binding site per molecule is assumed (Rosner & Bradlow, 1971). This value reflects only the polypeptide content of CBG, and if the carbohydrate content (23%) is taken into account a molecular weight of 58 238 can be calculated which is similar to the value obtained by SDS–PAGE or gel filtration. The range of CBG concentrations in normal men and women is small (14–28 mg/l), but we have observed individuals whose serum CBG concentration fell below this range (e.g. 8–6 mg/l; see Fig. 6). A similar observation was made by Lohrenz, Seal & Doe (1967), who discovered a familial deficiency of CBG.

These low serum concentrations of CBG are found in apparently normal individuals, in contrast to other pathophysiological situations, e.g. septic shock (Zouaghi, Savu, Delorme et al. 1983), where the CBG cortisol binding capacity appears to be very low. Serum concentrations of CBG generally increase in women taking various forms of oral contraceptives, almost to the same extent as observed during pregnancy, and this is thought to be due to the induction of hepatic CBG synthesis by oestrogens. Interestingly, the administration of different progestins (e.g. desogestrel or levonorgestrel) in combination with ethinyl-oestradiol does not influence the oestrogen-induced increase in serum CBG, whereas levonorgestrel suppresses increases in serum sex hormone binding globulin concentration under similar circumstances, while desogestrel does not (Hammond, Langley, Robinson et al. 1984).

In conclusion, an accurate, reproducible solid-phase RIA for human CBG has been developed which is suitable for the measurement of large numbers of serum or plasma samples. Immobilization of the antibody eliminates the need for additional reagents or incubations to precipitate antibody-bound complexes. This makes the assay simpler to perform, less time-consuming, and avoids possible interference at high lipid concentrations. In addition, adjustments in the amount of CBG-Ab-cellulose used in the RIA increases assay sensitivity and will allow measurements of CBG concentration in other body fluids, tissue culture media, or cellular and subcellular preparations, where CBG is expected to be found in very low concentrations.

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