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A LIQUID-PHASE IMMUNORADIOMETRIC ASSAY (IRMA) FOR HUMAN SEX HORMONE BINDING GLOBULIN (SHBG)†

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Summary—An immunoradiometric assay (IRMA) for sex hormone binding globulin (SHBG) has been developed in which an ^{125}I-labeled monoclonal antibody ([^{125}I]S_1B_5) and a rabbit anti-SHBG antiserum (RAb) are incubated in "liquid-phase" with standards or samples, and RAb-bound complexes are separated using donkey anti-rabbit IgG antibody-coated cellulose. This immunoassay technique is characterized by several advantages; the [^{125}I]S_1B_5 imparts additional specificity and obviates the requirement for pure SHBG; the use of excess reagents reduces incubation times and also improves assay performance and sensitivity, and incubation in "liquid-phase" conserves and increases the efficiency of the RAb. The assay measures only non-denatured SHBG and is not influenced by the presence of steroid at the binding site. Assay specificity was demonstrated by parallelism between dilutions of pure SHBG and different serum samples. The quantitative recovery of SHBG added to serum, and the agreement between specific activities of SHBG in pure standards and sera, confirm the accuracy of the method. The within and between assay coefficients of variation were $<7\%$ and $<11\%$, respectively, between 12 and 450 nmol/l. The assay sensitivity may be manipulated by altering the concentration of RAb and/or by preincubation with either [^{125}I]S_1B_5 or RAb, and 0.2 fmol SHBG may be measured on a standard curve. The SHBG assay has been used to measure SHBG concentrations in sera, amniotic fluid, cerebral spinal fluid, seminal plasma and saliva.

INTRODUCTION

Until recently, sex hormone binding globulin (SHBG) has been measured by a variety of steroid binding capacity assays [1–5], which rely on the assumption that its steroid binding characteristics are identical in different samples and that non-specific interactions between tracer ligand and other proteins are negligible. In addition, the steroid binding activity of SHBG is temperature and pH dependent [6, 7], and exogenous steroids and drugs may compete with the labeled ligand for the steroid binding site [8]. In order to circumvent these problems, various immunochemical methods have been developed to quantify SHBG directly, such as "rocket immunoelectrophoresis" [9], an enzyme immunoassay [10] and radioimmunoassays [11–13].

In comparison with other methods, radioimmunoassays for SHBG appear to be of equal or superior sensitivity and precision, and are more suited for routine analyses. The usefulness of this method has, however, been limited by the instability of pure SHBG [12, 13] as well as problems associated with its radioiodination and the subsequent purification and storage of [^{125}I]SHBG [12, 13]. Attention has therefore focused on the development of alternative methods which do not require pure SHBG as label or standard. Immunometric assays which employ labeled antibodies, such as the enzyme-linked immunosorbant assay (ELISA) recently described for SHBG [10], are particularly attractive in this respect because their characteristics and performance are similar to those of a radioimmunoassay (RIA), but their development has been hampered by the shortage of sufficient quantities of pure monospecific anti-SHBG immunoglobulins. It was largely for this reason that we produced and characterized a monoclonal antibody for human SHBG [14], and have now used it as the labeled probe in a "liquid-phase" immunoradiometric assay (IRMA) for the quantification of SHBG in biological samples.

EXPERIMENTAL

Reagents

The Bolton and Hunter reagent (*N*-succinimidyl 3-(4-hydroxy, 5-[^{125}I]iodophenyl) propionate; 2,000 Ci/mmol) and [1,2-^{3}H] 5α-dihydrotestosterone (51 Ci/mmol) were obtained from Amersham International plc, Bucks, U.K. When necessary the [^{3}H] 5α-dihydrotestosterone 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. Radioinert 5α-dihydrotestosterone

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†Also known as Testosterone-estradiol Binding Globulin (TeBG) and Sex-steroid Binding Protein (SBP).

(DHT), cortisol, microparticulate cellulose (Sigmacell type 20), dextran (av. mol. wt 70,000), Sephadex G75 IEF, Protein A-Sepharose CL 4B and 1-1′-carbonyl-diimidazole were from Sigma (London) Chemical Co. Ltd, Poole, U.K. and Norit A charcoal was from Amend Drug and Chemical Co., Irvington, NJ. Horse serum was obtained from Flow Laboratories, Irvine, U.K., and Sac-Cel™ (donkey anti-rabbit IgG antibody-coated cellulose) was from Wellcome Research Laboratories, Beckenham, U.K. All other laboratory reagents and solvents were of analytical reagent grade and were purchased from BDH Chemicals Ltd, Dorset, U.K. The assay buffer used throughout was 0.14 M phosphate buffered saline, pH 7.4, containing 0.1% gelatin and 0.1% sodium azide (PBS).

SHBG binding capacity assay

The binding capacity of SHBG for DHT was determined by a saturation analysis method [15] in which samples were diluted 1:100 in a solution of PBS containing 0.25% charcoal and 0.025% dextran (DCC–PBS), and incubated at 20°C for 30 min to remove endogenous steroids. After centrifugation (3000 *g* for 10 min) aliquots (100 μl) of the supernatants were incubated (1 h at 25°C) with 10 nM [^{3}H]DHT and 200 nM cortisol in the presence (non-specific binding) and absence (total binding) of 2 μM DHT. After a further incubation at 0°C (15 min), non-SHBG-bound steroids were removed by incubation (10 min at 0°C) with 500 μl DCC–PBS and centrifugation (3000 *g* for 10 min). The supernatants containing SHBG-bound [^{3}H]DHT were decanted into vials containing 2.5 ml Rialuma (Lumac B. V., Basle, Switzerland), and counted in a liquid scintillation spectrophotometer for 10 min or until 10,000 counts had accumulated. The SHBG binding capacity (nmol/l) was calculated from the amount of [^{3}H]DHT bound specifically to SHBG after correction for sample dilution. No correction was made for the recovery of the [^{3}H]DHT–SHBG complex during the DCC separation step, as this was <5%.

Purification of SHBG

Two approaches were used for the purification of human SHBG. The first was based on a combination of DEAE–ion exchange chromatography, affinity chromatography, and preparative polyacrylamide gel electrophoresis, as described by Petra and Lewis[16], but we were unable to produce monospecific antisera in rabbits using SHBG prepared in this way (see below). We therefore employed an alternative approach, similar to that described recently [17]. In brief, 1 l of human pregnancy serum was applied (60 ml/h) to a 2 × 2.6 cm column of 3-oxo-17β-hydroxy-5α-androstane-17α-(6-hexyn-1-ol) linked covalently to diaminoethyl-oxirane Sepharose CL 4B [16], the SHBG binding capacity of which was 40 nmol/ml gel. The affinity gel was then removed from the column and washed at 4°C with 3 × 10 ml 10 mM Tris–HCl buffer, pH 7.5, containing 1 M KCl (TK buffer), 2 × 10 ml TK buffer containing 10% dimethylformamide and 10 ml TK buffer containing 20% ethanol. The SHBG was then displaced from the gel by incubation (1 h at 20°C) with 10 ml TK buffer containing 20% ethanol and 1.7 mg DHT. The eluate was diluted 4-fold and concentrated to 1 ml by ultrafiltration through a PM10 filter in a 10 ml Amicon Ultrafiltration cell (Amicon Ltd, Bucks, U.K.). The concentrate was then applied to a horizontal, prefocussed (8 W constant power for 5 h at 10°C), Sephadex G75 IEF (5 g) preparative isoelectrofocussing gel (0.4 × 11 × 24.5 cm) using a polyampholyte (Serva Feinbiochemica GmbH & Co, Heidelberg, F.R.G.) gradient, pH 3–7, and focussed for a further 16 h under the same conditions. The gel was sliced (7 mm segments) and eluted with 8 ml 25 mM Tris–HCl, pH 8.5. Eluates from fractions that focussed above pH 5, and contained SHBG activity, were pooled and applied to a 12 × 0.9 cm DEAE-Sephacel column. The column was washed with 10 ml 25 mM Tris–HCl, pH 8.5, and eluted with a 0–0.3 M NaCl step gradient (10 mM NaCl/3 ml step) developed in the same buffer. Fractions containing SHBG were pooled and concentrated in an Amicon Minicon B15 concentrator and stored at −100°C.

The protein isolated in this way was considered pure by polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate (SDS)–PAGE, and by immunochemical analysis of antisera raised against it (see Results section). The mean ± SD protein concentration in a stock solution of pure SHBG was determined as 0.75 ± 0.04 mg/ml ($n = 4$) by the Bradford protein assay [18] in the presence of SDS (30 mg/l Bradford reagent), as recommended by Macart and Gerbaut[19], and using human serum albumin and bovine immunoglobulin as standards. The specific activity of SHBG (nmol DHT binding capacity/mg protein) in the stock solution was estimated as 8.8 nmol/mg protein, after it had been diluted 1:100 in DCC to remove DHT and further diluted (1:100) in PBS for analysis.

Anti-SHBG antisera

Purified SHBG was used for the production of antisera in rabbits, as described by Vaitukaitis *et al.* [20]. Initial subcutaneous, multiple site immunizations (100 μg SHBG emulsified in 1 ml Freund's complete adjuvant and 1 ml 0.9% NaCl) were followed by 3–5 booster immunizations (50 μg SHBG) at 2–3 weekly intervals. Ten days after the final booster, the rabbits were bled and antiserum was prepared, and stored at −20°C.

Titers were determined by incubating (16 h at 20°C) serial dilutions of antisera (100 μl in PBS) with 40,000 dpm [^{125}I]SHBG (100 μl), and immunoprecipitating antibody-bound [^{125}I]SHBG by incubation (1 h at 20°C) with 200 μl 4% second antibody (donkey anti-rabbit IgG) in 10% polyethylene glycol 4000 (PEG), and centrifugation (3000 *g* for 30 min).

Table 1. Titers, equilibration times, and dissociation-rate constants (K_d) of the rabbit anti-SHBG antisera (RAbs) shown in Fig. 1

RAb	Titer* (antiserum dilution)	Equilibration time† (h)	$K_{d\text{-}11}$ ($\times 10$ M)
No. 1	1:50,000	7	1.46
No. 2	1:24,000	10	1.43
No. 3	1:9000	10	1.16

*Determined as in Experimental and defined as the anti-serum dilution which yields 50% of the maximum binding of [^{125}I]SHBG.
†Time required to achieve maximum binding of [^{125}I]SHBG, using antisera dilutions shown under titer.

Titers were estimated at 50% of maximum binding (Table 1). The equilibration time of the interaction between [^{125}I]SHBG and various antisera was determined by incubating dilutions of antisera, which gave 50% maximum binding in the titer assay, at 20°C with 40,000 dpm [^{125}I]SHBG for various times between 1 and 24 h; separation of antibody-bound complexes was then achieved by incubation (15 min at 20°C) with 100 μl Sac-Cel™, centrifugation (3000 *g* for 5 min), and aspiration of the supernatants (Table 1). Affinities of the antisera for [^{125}I]SHBG (16 μCi/μg protein) were determined by Scatchard analysis [21] using Sac-Cel™ to separate antibody-bound complexes, as described above, and the dissociation-rate constants (K_d) are given in Table 1. The specificities of the antisera were assessed by immunoelectrophoresis according to Grabar and Williams[22] against human pregnancy sera as well as pure SHBG (Fig. 1).

Monoclonal antibody

The production and characterization of the monoclonal anti-SHBG antibody (S_1B_5) has been described in detail [14]. Essentially, antibody secreting hybridomas were produced by mixing 1×10^7 immune spleen cells from a Balb/C mouse with an equal number of X63-Ag8.653 myeloma cells, and fusing the cells in the presence of PEG. The resulting hybrid cells were cultured in HAT (hypoxanthine, aminopterin, thymidine) selection medium, and then cloned and subcloned by the limiting dilution technique. One particular hybridoma cell line produced high titers of an IgG_{2a} antibody which has a high affinity (K_d 0.38×10^{-11} M) for [^{125}I]SHBG and has been shown to immunoabsorb SHBG from serum [14]. Culture medium was harvested from cells grown to confluence, and the antibody was purified by protein A-Sepharose CL 4B affinity chromatography [14], concentrated and washed with 0.1 M borate buffer, pH 8.5, in an Amicon Minicon B15 concentrator, and stored at 0.6–0.8 mg/ml in 50 μl aliquots, at −20°C.

Radioiodination of SHBG and monoclonal antibody

Pure SHBG and S_1B_5 antibody were radioiodinated with the Bolton and Hunter reagent, as recommended by Amersham International plc. In general, 6–8 μg protein/10 μl 0.1 M borate buffer, pH 8.5, was added to 0.5 mCi *N*-succinimidyl 3-(4-hydroxy,5-[^{125}I]iodophenyl) propionate, and incubated at 0°C for 15 min. After the addition of 500 μl 0.1 M borate buffer, pH 8.5, containing 0.2 M glycine, and a further incubation (0°C for 15 min), ^{125}I-labeled proteins were purified by exclusion chromatography on a Sephadex G100 column (20 × 1 cm) that was pre-equilibrated and eluted with 50 mM phosphate buffer, pH 7.5, containing 0.25% gelatin as carrier protein. The fractions containing ^{125}I-labeled proteins were stored at −20°C in 200 μl aliquots containing 2% horse serum, and in this form appear to be stable for at least 2 months. Approximately 10–26% of the ^{125}I-label was incorporated, resulting in specific activities of 8–16 μCi/μg protein. When analysed by agarose gel electrophoresis the ^{125}I-labeled proteins migrated as single peaks of radioactivity which corresponded to the R_f values of the unlabeled proteins, and their purity was calculated to be >98%.

RIA for SHBG

A radioimmunoassay (RIA) for SHBG was initially developed using highly purified [^{125}I]SHBG, and a solid-phase SHBG antiserum (SHBG-Ab-cellulose) prepared by incubating (16 h at 20°C) 1 ml of a 1:5 dilution of rabbit anti-SHBG antiserum (RAb) in 50 M barbitone buffer, pH 8.0, with 200 μg carbodiimidazole-activated cellulose (Sigmacell 20), as described by Chapman and Ratcliffe[23]. In the RIA, samples were diluted 1:50 in PBS containing 1% horse serum (PBS + HS), and 100 μl aliquots of SHBG standards (dilutions of a pregnancy serum standard in PBS + HS, see below) or diluted samples were added to duplicated tubes containing 100 μl [^{125}I]SHBG (~40,000 dpm) and 100 μl solid-phase antibody slurry (20 μg SHBG-Ab-cellulose + 180 μg carrier cellulose/ml PBS buffer). The mixture was then incubated for 16 h at 20°C. After the addition of 2 ml 0.9% NaCl, the insoluble material was sedimented (2000 *g* for 5 min), and the supernatant containing unbound [^{125}I]SHBG was aspirated. The cellulose pellets were then counted in an LKB/Wallac Multigamma counter (LKB/Wallac Oy, Turku, Finland), for 1 min, or until 10,000 counts had accumulated. A standard curve was plotted using the spline curve fitting program supplied with the counter, and concentrations of SHBG in unknown samples (nmol/l) were determined from the standard curve.

Table 2. Mean ±SD concentrations and ranges of SHBG in samples of serum and other body fluids taken from normal volunteers and various patient groups

Sample type and subjects	Dilution in PBS	n	SHBG nmol/l $\bar{X} \pm$ SD (range)
Serum			
Men	1:100	20	23 ± 9 (11–44)
Men on testosterone* replacement	1:100	40	25 ± 10 (8–68)
Pre-menopausal women	1:100	32	53 ± 24 (18–110)
Post-menopausal women	1:100	39	49 ± 23 (14–115)
Women taking o.c. (A)‡	1:200	10	150 ± 33 (98–206)
Women taking o.c.‡	1:100	10	66 ± 16 (48–95)
Pregnant women[a] (B)	1:400	5	402 ± 172 (247–668)
Hirsute women	1:100	10	25 ± 10 (8–36)
Amniotic fluid (term):	1:50†	1	2
Cerebrospinal fluid:	1:2†	3	0.97 (0.05–1.04)
*Seminal plasma:**	1:5†	5	0.13 (0.10–0.17)
Saliva (mixed):			
Men	1:2†	4	0.021 (0.010–0.038)
Women	1:2†	4	0.047 (0.016–0.078)
Pregnant women	1:8†	4	0.279 (0.104–0.540)

The sample dilutions used for the IRMA are also shown.
*Infertility patients.
†Measured using pre-addition and incubation with $[^{125}I]S_1B_5$ to increase assay sensitivity as shown in Fig. 8.
‡(A) 30 μg ethinyl estradiol + 150 μg desogestrel. (B) 30 μg ethinyl estradiol + 150 μg levonorgestrel. a = third trimester.

IRMA for SHBG

The protocol used for the IRMA of SHBG in serum is as follows: Duplicate aliquots (100 μl) of a dilution series of a human pregnancy serum standard or pure SHBG (see below), and duplicate aliquots (100 μl) of unknown samples (diluted in PBS + HS, 1:100 for men and women, and 1:400 for pregnant women), were incubated with a mixture of 100 μl RAb (1:1000 dilution in PBS + HS) and 100 μl $[^{125}I]S_1B_5$ (~40,000 dpm) for 1 h at 20°C. A slurry (100 μl) of Sac-Cel™ was then added to all tubes and after 15 min 2 ml 0.9% NaCl was added and the tubes were centrifuged at 2000 *g* for 5 min. The supernatants were aspirated and the cellulose pellets were counted in an LKB/Wallac Multigamma counter for 1 min. A standard curve was plotted using the spline curve fitting program, and unknown concentrations were interpolated and expressed in nmol binding capacity/l or mg protein/l. The SHBG concentrations in other biological fluids could also be measured in the same way at appropriate dilutions, as given in Table 2.

Standards

The concentration of protein in a pure SHBG standard (see under Purification of SHBG) was determined using a modification of the Bradford protein assay [19], and was diluted appropriately in PBS + HS to give a standard range of 0.01–1.25 mg SHBG/l. In routine assays, however, we used serial dilutions (1:200–1:6400 in PB + HS) of a pregnancy serum sample shown by Scatchard analysis to have a DHT binding capacity of 413 nmol/l. These serum dilutions (2060–33 pmol SHBG/l), were made prior to use from 50 μl aliquots of this sample that were stored at −20°C. The 1% horse serum was included in the diluent buffer to prevent loss of protein at low dilutions, and was chosen for this purpose because it does not cross-react with the rabbit anti-SHBG antisera (data not shown) or the monoclonal antibody [14].

Samples

Blood samples were obtained from normal volunteers or patients, and serum or plasma were stored at −20°C. Saliva, amniotic fluid, seminal plasma and cerebral spinal fluid (CSF) were obtained from normal volunteers or patients after routine pathology had been performed, and were stored at −20°C.

RESULTS

Specificity

The ^{125}I-labeled monoclonal antibody used in the IRMA confers an additional level of assay specificity, and essentially identical standard curves and control values were obtained using two different rabbit antisera (RAb No. 1 and RAb No. 2) which exhibit large differences in specificity (Fig. 1). Evidence for the specificity of the IRMA was also obtained by a demonstration of parallelism between serial dilutions of different serum samples and the pure SHBG standard (Fig. 2). In addition, the concentration of SHBG in a serum sample containing a low SHBG concentration (5 nmol/l) was identical when measured by the IRMA at dilutions of 1:100, 1:50, 1:25, 1:10 or 1:5.

In order to demonstrate that the IRMA only measures active SHBG and does not detect the denatured protein, a pregnancy serum sample was heated at 60°C for 10, 20, 40, 60 and 90 min. The DHT binding capacities and IRMA concentrations

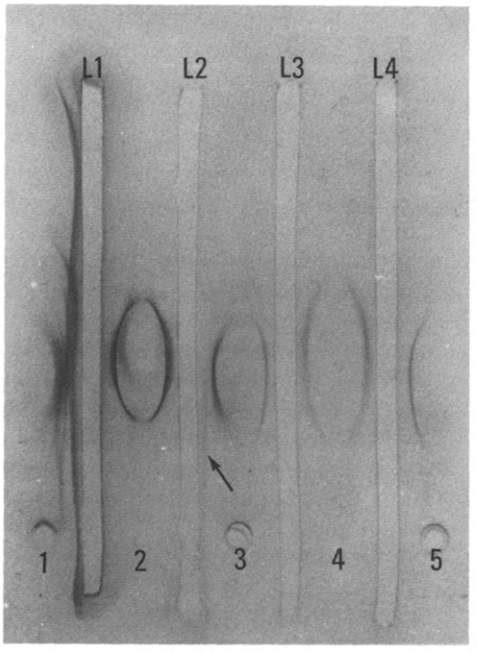

Fig. 1. The specificity of 3 rabbit anti-SHBG antisera (RAbs No. 1–3); assessed by immunoelectrophoresis [22] using 5 μl pure SHBG (0.75 mg/ml) in wells 2 and 4, and 20 μl pregnancy serum (wells 1, 3 and 5) as antigens. Lane (L) 1 contained100 μl RAb No. 1, L2 contained 100 μl RAb No. 2; L3 and L4 contained 100 and 200 μl RAb No. 3, respectively. The position of an immunoprecipitation arc, other than that associated with SHBG which develops between RAb No. 2 and pregnancy serum is indicated by the arrow. The RAb No. 3 was produced against SHBG purified by the method described, others were obtained by the method in [16] with (RAb No. 2) and without (RAb No. 1) the use of preparative PAGE.

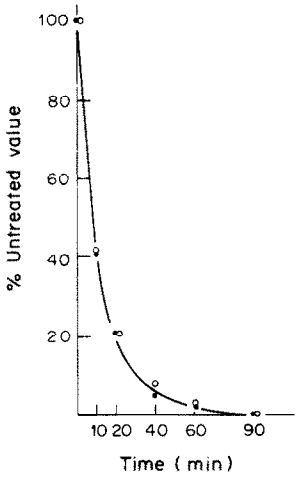

Fig. 3. Time-dependent effect of heat-treatment at 60°C, on the SHBG concentration in a late pregnancy serum, as measured by IRMA (○), and DHT binding capacity assay (●) [15]. The values at each time point are expressed as percentage of the value measured in the untreated sample by IRMA (254 nmol/l) and DHT binding capacity assay (284 nmol/l).

were then determined and compared with the values in the untreated samples (Fig. 3). The close agreement between the values obtained by both assays, at all time points, indicates that the IRMA measures only SHBG molecules which retain their steroid binding activity. To determine whether the loss of recognition of heat-denatured SHBG is entirely attributable to the specificity of the monoclonal antibody, we used heat-treated (60°C for 1 h) [^{125}I]SHBG (~30,000 dpm) to titer the monoclonal antibody and polyclonal RAbs. The results clearly indicate that the monoclonal antibody does not recognize heat denatured SHBG, while some recognition is retained by RAb No. 2 (Fig. 4). Other RAbs assessed in this way also behaved like RAb No. 2.

The mean ±SD concentrations of SHBG in two serum samples was also measured ($n = 6$) by IRMA before (30 ± 1 nmol/l and 111 ± 13 nmol/l) and after (31 ± 1 nmol/l and 116 ± 13 nmol/l) treatment (30 min at 20°C) with DCC to remove endogenous steroids [15]. This clearly shows that the IRMA

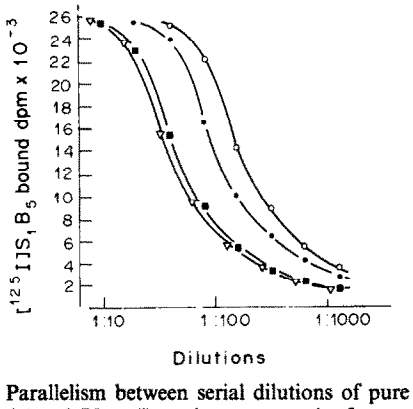

Fig. 2. Parallelism between serial dilutions of pure SHBG standard (▽, 0.75 mg/l), and serum samples from a normal man (■), woman (○) and woman during late pregnancy (●). The pregnancy serum was pre-diluted 1:10, and all dilutions were made in PBS + 1% horse serum.

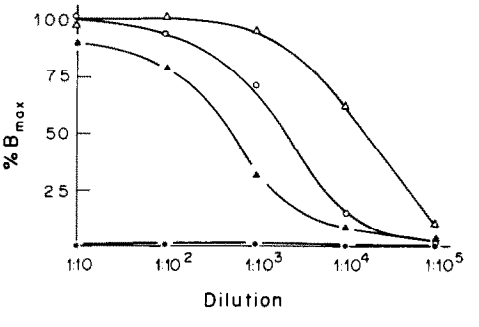

Fig. 4. Titers of the monoclonal antibody in culture medium (circles) and RAb No. 2 (triangles) using untreated (open symbols) and heat-treated (closed symbols) [^{125}I]SHBG (30,000 dpm). Specific binding of untreated and heat-treated (60°C for 1 h) [^{125}I]SHBG is expressed as a percentage of the maximum binding (B_{max}) obtained by incubating untreated [^{125}I]SHBG with a 1:10 dilution of S_1B_5 culture medium or RAb No. 2, as appropriate.

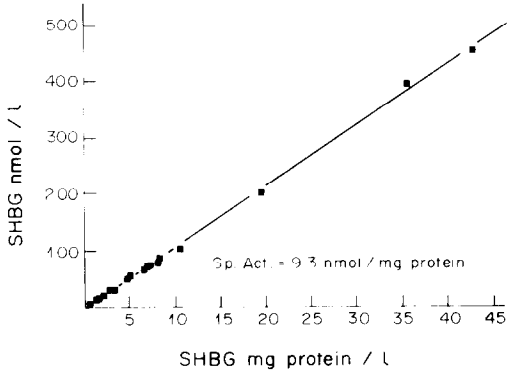

Fig. 5. Comparison of SHBG concentrations in serum samples ($n = 20$) measured by IRMA using pure SHBG standards (mg protein/l), or dilutions of a standard pregnancy serum calibrated in terms of its DHT binding capacity (nmol/l) by Scatchard analysis [21]. The relationship between the values obtained using these two standards is $y = 10.7x - 3.5$ ($r = 0.99$), and was used to calculate the specific activity of SHBG in the serum samples.

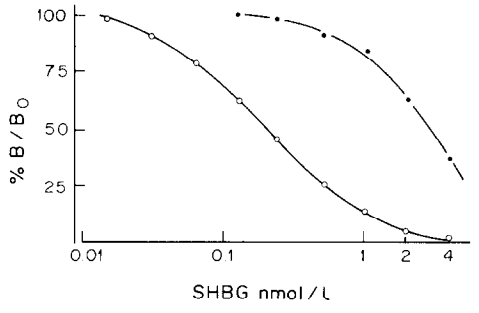

Fig. 6. Solid-phase SHBG–RIA standard curves generated using serial dilutions of a pregnancy serum standard (413 nmol/l) and RAb No. 1-cellulose (20 mg/ml PBS) diluted 1:4 (●) or 1:100 (○) with uncoupled cellulose (20 mg/ml PBS). Total amount of [^{125}I]SHBG added to each tube was 40,230 dpm, and B_0 at 1:4 RAb No. 1-cellulose was 32,650 dpm, while B_0 at 1:100 RAb No. 1-cellulose was 8,620 dpm.

measurement is independent of the presence of steroid at the SHBG binding site.

Accuracy

The accuracy of the IRMA was ascertained by the quantitative recovery of known amounts of pure SHBG added to a serum sample. The protein content and specific activity of a pure SHBG standard were determined (see under *Purification of SHBG*), and known amounts (0–3.3 pmol/50 μl) were added to 50 μl aliquots of a sample containing a very low concentration of SHBG (7 nmol/l). The concentrations of SHBG in the samples were then measured by the IRMA. When the values obtained were compared with the amounts of pure SHBG added, a mean recovery $\pm$SD of 91 $\pm$ 6% ($n = 5$) was calculated over the concentration range studied.

In addition, when serum SHBG concentrations ($n = 20$) were determined by the IRMA using pure SHBG as the standard, and the values were compared with those obtained using the pregnancy serum standard dilutions, the specific activity (9.3 nmol/mg protein) obtained (Fig. 5) is very similar to the value recorded for the pure SHBG standard (8.8 nmol/mg protein).

Sensitivity

In order to compare the range and sensitivity of a conventional RIA (Fig. 6) with those of the IRMA (Fig. 7), standard curves were generated using different concentrations of the same RAb. These data indicate that the ranges of both types of assay are similar but that the signal to noise (background dpm) ratio of the IRMA is greater than that achieved by the RIA, when similar amounts of the respective ^{125}I-labeled tracers are used. In addition, if sensitivity is defined as the mean + 2SD of the amount of ^{125}I-label in tubes containing assay buffer in place of

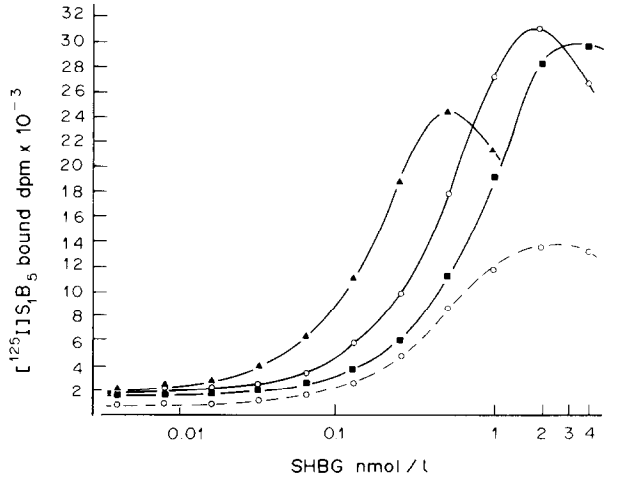

Fig. 7. Liquid-phase SHBG–IRMA standard curves generated using serial dilutions of a pregnancy serum standard (413 nmol/l), together with 1:500 (■), 1:1,000 (○) or 1:5,000 (▲) dilutions of RAb No. 1, and 50,000 dpm [^{125}I]S_1B_5 (solid lines), or a 1:1,000 dilution of RAb No. 1 and 25,000 dpm [^{125}I]S_1B_5 (broken line). The [^{125}I]S_1B_5 and RAb No. 1, were added simultaneously, and incubated for 1 h at 20°C, prior to the addition of Sac-Cel™.

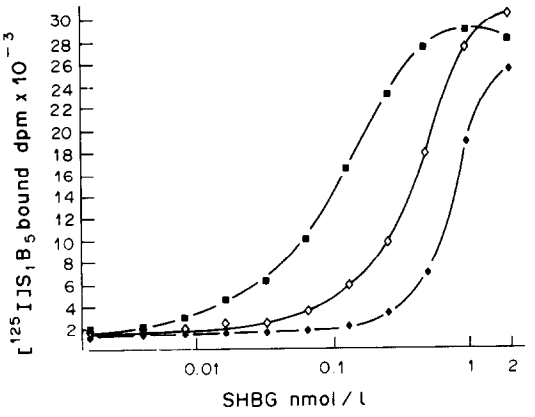

Fig. 8. Effect of altering the experimental protocol on the liquid-phase SHBG–IRMA standard curves generated using serial dilutions of a pregnancy serum standard (413 nmol/l), 1:1,000 dilution of RAb No. 1, and 50,000 dpm $[^{125}I]S_1B_5$. Simultaneous addition of $[^{125}I]S_1B_5$ and RAb No. 1 and incubation for 2 h at 20°C (◇); pre-addition of $[^{125}I]S_1B_5$ and incubation (1 h at 20°C) prior to addition of RAb No. 1 and a further 1 h incubation at 20°C (■); pre-addition of RAb No. 1 and incubation (1 h at 20°C) prior to addition of $[^{125}I]S_1B_5$ and a further 1 h incubation at 20°C (◆).

standard, the IRMA is able to detect as little as 0.2 fmol/assay tube, whereas 0.5 fmol/assay tube is the limit of sensitivity of the RIA. In the RIA, the assay sensitivity is also reduced by increasing the amount of labeled analyte, whereas the use of greater amounts of $[^{125}I]S_1B_5$ increases the IRMA sensitivity; for example, a 2-fold increase in sensitivity was observed when 50,000 dpm $[^{125}I]S_1B_5$ was used, as compared to 25,000 dpm (Fig. 7).

It was also found that pre-addition and incubation (1 h at 20°C) with $[^{125}I]S_1B_5$ increased assay sensitivity, while a decrease in sensitivity occurred if a similar preincubation with RAb was conducted prior to the addition of $[^{125}I]S_1B_5$ and a further incubation (1 h) at 20°C (Fig. 8).

Precision

Repeated duplicate measurements ($n = 5$) of serum samples containing low (12 ± 0.3), medium (36 ± 2.2), high (126 ± 2.6) and extra-high (450 ± 8.2) concentrations of SHBG (mean ± SD nmol/l) were made to assess the intra-assay variability of the IRMA. Expressed as coefficients of variation, these are 3, 6, 2 and 2%, respectively. Inter-assay ($n = 7$) coefficients of variation of measurements of the same samples were 10, 8, 7 and 11%, respectively.

Measurement of SHBG

The serum SHBG concentrations (nmol/l) in 43 men and 75 women were measured by the DHT binding capacity assay (BCA) and the IRMA. When the results of both assays were compared a correlation coefficient of 0.99 was obtained, and the equation describing the relationship between the BCA (x) and IRMA (y) results is $y = 0.92x + 1.05$. The serum concentrations of SHBG measured by the IRMA are also presented in Table 2 according to the physiological status of the subjects studied. We were also able to detect SHBG in various other biological fluids including saliva, CSF, amniotic fluid and seminal plasma. Dilutions of each sample were tested for parallelism against the IRMA standard, and measurable concentrations of SHBG were found in all samples tested (Table 2).

DISCUSSION

It is widely accepted that SHBG is a plasma transport protein for sex-steroid hormones and that its concentration determines the distribution of testosterone and estradiol between the various steroid binding proteins and the non-protein-bound fraction in the blood [24]. This is of particular importance, as it is thought that SHBG-bound steroids are not available to target cells and that only the albumin-bound [25] and/or non-protein-bound [26] fractions are "biologically active". Moreover, the presence of SHBG reduces the metabolic clearance rate of testosterone [27], and alterations in SHBG concentration may consequently influence total plasma sex-steroid hormone concentrations. For these reasons, attempts have been made to devise a simple and reliable method for the measurement of SHBG in blood samples. Recently, attention has focused on immunochemical methods since they promise to yield more sensitive and specific measurements that are not susceptible to the methodological limitations of steroid binding capacity assays, and are not influenced by unknown factors that inhibit or disrupt the steroid binding activity of the protein. The results obtained by published immunochemical assays agree and correlate well with those obtained by established binding capacity assays [10–13], but they offer little advantage in terms of assay simplification, precision, or the time required to obtain results. Our aim, therefore, has been to develop a simple assay based on the use of stable reagents which permits accurate and precise measurements in the shortest possible time.

The specificity of the IRMA is largely attributable to the use of a monoclonal antibody which, unlike the rabbit antisera we have produced, does not recognize denatured SHBG and appears to interact with an unique antigenic determinant that reflects its conformational structure. Therefore, in comparison with some immunochemical assays [10], fragments or denatured forms of SHBG are not measured by the IRMA. This is important because the binding activity of the protein is dependent on its conformational integrity [28]. Although such a high degree of specificity may not be essential for measurements of SHBG in blood samples, it ensures the detection and quantification of conformationally intact SHBG molecules in biological fluids and tissue extracts in which the protein may have been denatured by proteolytic

enzymes or other cellular constituents. In addition, steroid occupancy of the SHBG steroid binding site does not influence the results, and it therefore appears that the monoclonal antibody does not recognize this region of the molecule. This may be technically advantageous but it is possible that variants of SHBG exist with an abnormality in the steroid binding site, and measurements of concentration by the IRMA or any other immunochemical assay may not reflect the true biological activity of such variants, especially if their affinity for steroids is abnormal. At present, there have been no reports of discrepancies between immunoassay and steroid binding capacity measurements of SHBG, and this has been confirmed in the present study and by measurements of many other normal and pathological samples (unpublished observations) by both IRMA and a DHT binding capacity assay [15]. Nevertheless, although electrophoretic variants of SHBG have been reported [29] it is not known whether they reflect abnormalities in carbohydrate or peptide composition, or if they exhibit any defects in steroid binding activity.

The accuracy of the IRMA has been confirmed by comparison with an alternative method, i.e. the DHT binding capacity assay [15], and we have demonstrated the quantitative recovery of SHBG added to a serum sample. In addition, when pure SHBG standards were prepared in terms of their protein concentration and used in the IRMA, the values obtained (mg protein/l) correlated well ($r = 0.99$; $n = 20$) with IRMA values (nmol/l) obtained using a serum standard (calibrated in terms of its DHT binding capacity by Scatchard analysis). The specific activity recorded is very similar to that of the pure SHBG standard, and this indicates that the steroid binding activity of the pure SHBG standard is identical to SHBG in serum samples. Moreover, the specific activities obtained agree with estimates of the molecular weight (88,000–115,000 Daltons) of dimeric SHBG [28, 30, 31] if one steroid-binding site per dimer is assumed.

The sensitivity of the IRMA is almost 2 orders of magnitude greater than the most sensitive steroid binding capacity assays [e.g. 2, 15] and appears to be marginally more sensitive than the solid-phase RIA developed for comparison; an assay which is as sensitive as other RIAs described recently [13, 32]. The greater signal to noise ratio of the IRMA probably contributes to its greater sensitivity. Moreover, in direct contrast to conventional RIAs, increases in the amount of tracer used in the IRMA improves assay sensitivity. The noise experienced in both immunoassays was generally $< 5\%$ of the total activity used, and appears to be highly dependent on the purity and stability of the ^{125}I-labeled proteins. In our hands, the Bolton and Hunter reagent has always provided ^{125}I-labeled SHBG and monoclonal antibody which require no purification other than the initial separation of uncoupled reagent, and are stable for several months.

In comparison with other immunochemical assays, the IRMA is characterized by several technical advantages. The use of a labeled monoclonal antibody obviates the requirement for pure SHBG or monospecific anti-SHBG antisera. Incubation times have been reduced by the use of excess reagents, and results may be obtained within 2 h as opposed to 1–2 days [12–13]. The use of excess reagents also reduces the need for precise pipetting, and thereby improves the assay performance in the hands of a novice. Although the RAb may be coupled directly to a solid-phase, large amounts of anti-SHBG antisera are consumed in the process and the antibodies appear to be generally less efficient when immobilized. In addition to conserving supplies of RAb, the performance of the immunoradiometric reaction in "liquid-phase" also ensures that the amount of RAb added may be controlled precisely within the between assays. The only disadvantage we have encountered with this type of protocol is the "hook-effect" at high analyte concentration. This is a characteristic of all IRMAs in which "labeled antibody" and "immobilizing antibody" are present simultaneously, and is due to the saturation of "immobilizing antibody" with analyte. In practice, this problem is circumvented by increasing the concentration of immobilizing antibody, or by adjusting the sample dilutions so that the anticipated analyte concentration falls within the range of the standard curve.

The serum concentrations of SHBG measured by the IRMA agree with those recently obtained by RIA [13] and various binding capacity assays [1–5, 15]. In addition, the SHBG concentration in a term amniotic fluid is within the range of DHT binding capacity values measured in samples taken during late pregnancy [33]. Of perhaps greater interest is the detection and measurement of SHBG in CSF, seminal plasma and saliva. As is the case with many other plasma proteins, the presence of SHBG in CSF can be attributed to non-specific permeability of the "blood–brain" barrier. In this regard, the samples we analysed were from patients who were subsequently diagnosed as normal and the SHBG concentrations measured are approx 2% of expected plasma SHBG concentrations; a value which is very similar to the normal CSF: plasma ratio of albumin concentrations. Recently, immunochemical data have indicated that an androgen binding protein (ABP) which shares many of the physicochemical characteristics of SHBG is present in tissues from the testis and epididymis [34,35], but until now there have been no reports of measurements of SHBG or ABP in seminal plasma. Previously SHBG and ABP have been distinguished by virtue of their binding affinity to the lectin concanavalin A [34], and preliminary (unpublished) data indicate that the IRMA measurements of SHBG in seminal plasma are associated only with the typical "SHBG-like" concanavalin A binding-form of these proteins. The samples studied were obtained from infertile men and it remains to be

seen if the values recorded are similar to those in seminal plasma from normal men. We have also been able to identify and measure SHBG in mixed saliva samples from normal men and women, and in general the salivary SHBG content is approx 0.1% of plasma levels. This observation warrants a more detailed study to establish the source and stability of SHBG in saliva, because its presence may influence the salivary concentrations and/or measurements of some steroids under certain conditions. It is also anticipated that the sensitivity of the IRMA will permit measurements at the cellular and subcellular level, and facilitate studies designed to elucidate the hormonal regulation of SHBG synthesis by cells in culture.

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