Time-Resolved Immunofluorometric Assay of Sex-Hormone Binding Globulin

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A time-resolved immunofluorometric assay (trIFMA) for human sex-hormone binding globulin (SHBG) is described in which antibody-coated tubes or microtiter strip-wells and a europium (Eu) chelate-labeled monoclonal antibody are used. The trIFMA sensitivity is similar to that of other SHBG immunoassays, and other analytical variables compare favorably with an SHBG immunoradiometric assay (IRMA) kit and a steroid binding capacity assay: the interassay coefficient of variation (CV) is <8% and the intra-assay CV is <6% for concentrations between 6 and 200 nmol/L. The reference intervals (means ± SD) for SHBG concentrations (nmol/L) in serum from 10 men, 10 women, and 10 pregnant women were 23 ± 12, 65 ± 39, and 439 ± 122, respectively. In 14 hirsute women the mean ± SD serum SHBG concentration (37 ± 21 nmol/L) was significantly lower (P < 0.01) than the mean for an age-matched, nonhirsute female comparison group. The trIFMA is technically simple, requires no centrifugation or separation reagent, and takes a counting time of only 1 s. In addition, the Eu-label is nontoxic, presents no waste-disposal problems, and has a long shelf life.

Additional Keyphrases: chelated europium label · monoclonal antibodies · reference interval · pregnancy · hirsutism

Sex-hormone binding globulin (SHBG) is a glycoprotein in plasma that has a high affinity for testosterone and estradiol, and appears to regulate the bioavailability of these steroid hormones (1). Until recently, SHBG has been quantified by various assays of steroid binding capacity (2–6), which rely on the assumptions that its steroid-binding characteristics are identical in different samples, and that nonspecific interactions between tracer ligand and other proteins are negligible. However, the steroid-binding activity of SHBG is influenced by temperature and pH (7, 8), and exogenous steroids and drugs may compete with labeled ligand for the steroid binding site (9). To circumvent these problems, workers have developed several immunochemical methods to quantify SHBG directly (10–15). Compared with other methods, radioimmunoassays for SHBG appear to be of equal or superior sensitivity and precision, but their use has been limited by the instability of pure SHBG and by problems associated with its radioiodination (11, 12). Attention has therefore been focused on development of immunological methods of binding capacity assays involving labeled antibodies (14–16). Ideally, the label should be nonhazardous, provide a good signal-to-noise ratio, and have a long shelf life. The introduction of lanthanide chelates to generate labeled fluorescent probes promises to satisfy these criteria (17), and we have therefore used a chelated europium (Eu) label to develop a time-resolved immunofluorometric assay (trIFMA) for routine determination of SHBG concentrations in blood samples.

Materials and Methods

Materials. The assay buffer was Tris HCl (50 mmol/L, pH 7.75) containing, per liter, 9 g of NaCl, 5 g of bovine serum albumin, 0.5 g of sodium azide, 0.5 g of bovine immunoglobulin, 0.1 g of Tween 40 (polyoxyethylene (40) sorbitan monolaurate), and 20 μmol of DTPA. The 50 mmol/L Tris-buffered saline wash solution, pH 7.75, contained the bacteriostatic agent "Germall II" (Chemag, Frankfurt/Main, F.R.G.; 1 g/L) and Tween 20 (0.05 g/L). Enhancement solution was used as supplied (Wallac Oy, Turku, Finland); it contained, per liter, 1 g of Triton X-100, 6.8 mmol of potassium hydrogen phthalate, 100 mmol of acetic acid, 50 μmol of tri-n-octylphosphine oxide, and 15 μmol of 2-naphthoyltrifluoroacetate.

Test tubes (11.5 × 75 mm; Sarstedt, Rommendorf, F.R.G.) and strip-wells (0.3 mL per well; Labsystems, Helsinki, Finland) coated with the immunoglobulin fraction of a polyclonal antiserum raised in rabbits against SHBG were from Farmos Diagnostica Ltd., Oulunsalo, Finland. The dry tubes and strip-wells were stored in air-tight containers at 4 °C until use.

Eu-labeled anti-SHBG monoclonal antibody. Monoclonal mouse antibodies against SHBG were purified from cell-culture medium as described previously (18). We reacted 1 to 3 mg of this antibody with a 160-fold molar excess of N'-[5-iodo-2-(trifluoromethyl)-benzene-1-sulfonamido]diethylenetriaminepentaaetic acid Eu chelate (Wallac Oy) for 18 h at 4 °C. We separated the Eu-labeled antibody from excess reagent by gel filtration on a two-tier column (1.5 cm i.d.) of Sephadex G25 (5 cm deep) and Sepharose 6B (30 cm deep), eluting with 50 mmol/L Tris HCl-buffered saline (pH 7.75) containing sodium azide (0.5 g/L). We collected 1-mL fractions and monitored them for fluorescence and for ultraviolet absorbance at 280 nm. Fractions containing the labeled antibody were pooled and DTPA-purified bovine serum albumin was added to give a final concentration of 10 g/L. The pool, filtered through a 0.2-μm sterile filter and stored at 4 °C, was usable for at least nine months. With this labeling procedure, about 10 molecules of Eu were attached per antibody molecule.

Apparatus. A Model 1230 "Arcus" fluorimeter, designed specifically for time-resolved fluorometry, was used (LKB-Wallac, Turku, Finland). We also used manual and automatic aspirating/washing systems (LKB-Wallac) compatible with the strip-wells, and an automatic shaker (LKB-Wallac) for mixing the contents of the strip-wells or tubes.

Blood samples. Blood was sampled from 120 anonymous blood donors (Pinnish Red Cross). Blood samples were also taken from 10 women during the third trimester of pregnancy, from 14 untreated women (ages 25–42 y) who were classified clinically as hirsute, and from 14 age-matched healthy women (ages 26–42 y). Serum was separated and stored at -20 °C.

SHBG binding capacity assay. The binding capacity of

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4 Nonstandard abbreviations: SHBG, sex-hormone binding globulin (also known as testosterone-estradiol binding globulin and sex-steroid binding protein); trIFMA, time-resolved immunofluorometric assay; IRMA, immunoradiometric assay; DTPA, diethylenetriaminepentaaetic acid; and DHT, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one).

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SHBG for 5α-dihydrotestosterone (DHT) was determined by a saturation-analysis method, which relies on the use of 3H-labeled DHT as tracer and dextran-coated charcoal as separation reagent (19). The SHBG binding capacity (nmol/L) was calculated from the amount of 3H-DHT specifically bound to SHBG after correction for sample dilution, with no correction for dissociation of the 3H-DHT–SHBG complex during the separation step, which was <5%.

**SHBG immunoradiometric assay (IRMA).** For comparison, the concentration of SHBG in serum samples was also determined with a commercially available kit (Farmos Diagnostica Ltd.) based on a liquid-phase IRMA (15). The assay was performed without modification according to the manufacturer’s instructions.

**SHBG trIFMA.** Dilute serum standards, control sera (low and high concentrations), and samples 100-fold (400-fold for pregnancy sera) in assay buffer and vortex-mix. Pipet duplicate 25-μL aliquots of the diluted standards and samples into anti-SHBG antibody-coated wells or tubes. Dilute the anti-SHBG–Eu tracer in assay buffer (~10⁷ counts/s per 200 μL) within 1 h of use, and add to the wells or tubes. Shake the samples for 2 min (automatic shaker), then incubate for 2 h at room temperature. Aspirate or decant the incubation mixtures, rinse the tubes or wells several times with wash solution, and add enhancement solution to each well (200 μL) or tube (600 μL). Shake the containers for 5 min, then let them stand for 10–15 min before measuring the fluorescence (1 s per well or tube).

**Standards.** The standards used in the IRMA and trIFMA were lyophilized aliquots (0.5 mL) of serial dilutions (two- to 64-fold in fetal bovine serum) of a human pregnancy serum pool that was shown by Scatchard analysis (19, 20) to have a DHT-binding capacity of 413 nmol/L. The “zero” standard was fetal bovine serum alone. All standards also contained lactose (25 g/L) and sodium azide (1 g/L) as preservative, and were reconstituted with 0.5 mL of distilled water prior to use. The reconstituted standards were stable for at least three weeks when stored at 4 °C.

**Results**

**Specificity.** The specificity and other characteristics of the monoclonal antibody used in the present assay have been described previously (15, 18). In an additional test, we heated a specimen of pregnancy serum at 60 °C for up to 90 min, then compared the DHT-binding capacity (19) and trIFMA measurements at various times with the results obtained for the unheated serum. The parallel time-dependent decrease in the values obtained by both assays indicated that the trIFMA measures only SHBG molecules that retain their steroid-binding activity (Figure 1).

**Sensitivity.** Whether strip-wells or tubes are used, the sensitivity (least detectable amount) of the IFMA for SHBG, defined as the point equivalent to the mean ± 3 SD antibody bound (counts/s) at zero ligand concentration, is 8 pmol per liter of diluted serum sample, equivalent to 0.2 fmol per assay well or tube. Figure 2 illustrates a typical dose–response curve and precision profile (based on strip-wells). Use of the same Eu-labeled antibody solution in wells and tubes gave a 30% lower final signal (controls) in the tubes. However, the slopes of the standard curves were practically identical. When tubes were used, imprecision (CV) remained <5%.

**Analytical recovery.** Known volumes of a pregnancy serum pool containing 254 nmol of SHBG per liter were added to a serum sample containing a low (9 nmol/L) concentration of SHBG. The mean (n = 10) recovery was 100% (SD to 10.5%), with measurements of SHBG concentrations ranging from 11 to 130 nmol/L.

**Precision.** To assess the intra-assay variability of the trIFMA with strip-wells, we made repeated duplicate measurements (n = 10) of serum samples containing low (10–24 nmol/L, n = 6), medium (31–89 nmol/L, n = 11), or high (111–156 nmol/L, n = 9) concentrations of SHBG. The mean CVs for these three groups were 4.8%, 5.2%, and 5.8%, respectively. Inter-assay (n = 10) CVs of measurements of the same samples were 6.4%, 5.1%, and 7.3%, respectively.
Precision data were essentially identical when tubes were used.

Measurements of SHBG in serum. The concentrations of SHBG in serum for healthy men, women, and women during pregnancy are summarized in Table 1. We also measured the concentrations of SHBG in serum from 100 blood donors by triFMA (x) and IRMA (y). The equation describing the regression line was y = 1.15x - 2 nmol/L (r = 0.99). When the data were compared on the basis of sex, the regression equation for samples from men (n = 50) was y = 1.10x + 1 nmol/L (r = 0.98), and y = 1.16x - 5 nmol/L (r = 0.99) for samples from nonpregnant, nonhirsute women (n = 50). SHBG concentrations (mean ± SD) measured in serum from 14 untreated hirsute patients were 37 ± 21 nmol/L (range 13–81 nmol/L) by triFMA and 33 ± 20 nmol/L (9–81 nmol/L) by IRMA. The triFMA values were significantly lower (P < 0.01) than those obtained for 14 age-matched healthy women (61 ± 22 nmol/L). Results for the patients’ samples also showed a good correlation between the values obtained by triFMA (x) and IRMA (y); y = 0.96x - 3 nmol/L (r = 0.97).

We deliberately hemolyzed five samples by passing them through a 27-gauge needle, then analyzed the resulting serum for SHBG by triFMA. The mean SHBG concentration in the hemolyzed samples (60.0 nmol/L) was practically identical to that for the serum prepared from nonhemolyzed blood taken from the same subjects at the same time (59.5 nmol/L). Likewise, mean concentrations of SHBG measured by the triFMA in extremely turbid lipemic sera (n = 4) were very similar before (21.7 nmol/L) and after (18.0 nmol/L) treatment with Lipoclean™ detergent (Behring Institute, Marburg, F.R.G.).

Discussion

The simplicity and speed of the triFMA make it ideal for quantifying serum SHBG in clinical laboratories. In comparison with an established IRMA for SHBG (15), the need for second-antibody separation is obviated by coating the assay tubes or wells with an excess of rabbit anti-SHBG antibodies. By thus "catching" the SHBG–Eu-labeled monoclonal antibody complexes that form during the incubation, one can replace a centrifugation step with a more rapid and cost-effective step of washing/aspiration or decantation to separate the free from the bound complexes.

The excellent signal-to-noise ratio obtained in the triFMA contributes to the high sensitivity and precision of the assay. Although the low concentrations measurable in this assay (0.2 fmol/assay well or tube) are not required for routine analyses of SHBG concentrations in blood samples, it may prove valuable for studies of SHBG production in cell cultures, or for measurements in other body fluids or tissues. Other characteristics of the triFMA compare favorably with those of established immunochemo and steroid binding capacity assays for SHBG. Moreover, it appears that, like the IRMA (15), the triFMA detects only SHBG molecules that possess steroid binding activity.

Measurements of SHBG concentrations in serum have been used to identify and characterize several clinical disorders related to inappropriate sex-hormone activity in men and women (21). The most common of these is hirsutism, associated with excessive androgenic action on the hair follicle. When this condition can be attributed to an increase in non-protein-bound testosterone, caused by low concentrations of SHBG in blood (1, 21), affected women can be administered oral contraceptives high in estrogen, to increase their SHBG concentrations in plasma and thereby reduce the "bioavailability" of endogenous androgens (21, 22). Measurements of SHBG in serum are also often used to monitor the effectiveness of this type of treatment.

In addition to estrogen, one of the most potent inducers of hepatic SHBG synthesis is thyroid hormone (21). Thus some laboratories also advocate the use of serum SHBG measurements as an index of thyroid hormone action, and claim that it is especially useful in the diagnosis of familial thyroid hormone resistance (23).

In comparison with the use of radiolabeled tracers, the chelated Eu-labeled monoclonal antibody is nontoxic, presents no waste-disposal problems, and has a relatively long shelf life. Moreover, because of its stability, large batches of this reagent may be prepared at one time. This is particularly advantageous, eliminating the constant task of performing radiodinations and the costly disposal of radioactive waste.

We conclude that this triFMA of SHBG will be a useful addition to the growing number of time-resolved immunoassays being developed for clinical use. In particular, it should provide a valuable adjunct to immunoassays of circulating testosterone and estradiol concentrations, for clinical evaluations of the bioavailability of these hormones in patients who present with symptoms of inappropriate sex-steroid hormone activity.

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| Table 1. Concentrations of SHBG in Serum of Men, Women, and Women during Late Pregnancy |
|---------------------------------|---|---|---|
| SHBG concn, nmol/L             | Mean | SD | Range     |
| Men (n = 10)                   |     |   |           |
| triFMA                         | 23  | 12 | 8–46      |
| IRMA                           | 26  | 14 | 8–51      |
| DHT-BCA                        | 28  | 16 | 10–55     |
| Women (n = 10)                 |     |   |           |
| triFMA                         | 65  | 39 | 21–127    |
| IRMA                           | 68  | 43 | 22–140    |
| DHT-BCA                        | 71  | 42 | 24–143    |
| Late pregnancy (n = 10)        |     |   |           |
| triFMA                         | 439 | 122| 267–610   |
| IRMA                           | 469 | 138| 295–677   |
| DHT-BCA                        | 423 | 122| 257–594   |

*The present assay (trIFMA), IRMA (Farmos Diagnostica), and a DHT-binding capacity assay (DHT-BCA; ref. 19).

All correlations within groups were highly significant (P < 0.001).
References