Particle-Enhanced Turbidimetric Immunoassay of Sex-Hormone-Binding Globulin in Serum
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A particle-enhanced turbidimetric immunoassay (PETIA) for human sex-hormone-binding globulin (SHBG) is described. The method involves use of antibody covalently coupled to latex particles and is almost fully automated, with sample processing being complete in <20 min. The working reagents are stable for at least three months, and full calibration of the assay each day is not essential. A particular advantage is that pretreatment of samples is rarely required because the working range of the assay is from 2.0 to 320 nmol/L for nondiluted serum. Intrassay CVs were <4.5% and 8.5%, respectively, and mean analytical recovery was 101.5%. SHBG concentrations of 129 serum samples determined by this method and by a commercially available immunoradiometric assay correlated highly.

Additional Keyphrases: latex agglutination · immunoradiometric assay compared

The free androgen index (FAI), derived from parallel measurements of plasma testosterone and sex-hormone-binding globulin (SHBG), is recommended as a sensitive indicator of androgen status (1,2). For this and other reasons, the routine measurement of SHBG in serum is increasing (3). Until recently, SHBG could be measured only by its binding of radiolabeled steroids, and many binding assays have been described (4). However, these are technically demanding and are affected by temperature (5), exogenous steroids and drugs (6), and the procedures used to isolate specifically bound steroids (7).

Immuoassays have overcome these problems but have introduced others. Electroimmunodiffusion is laborious and requires relatively large amounts of antisera (7); radioimmunoassay is disadvantaged by the necessity for pure SHBG and difficulty in radiolabeling the protein (8). Among methods involving labeled antibodies (9–12), immunoradiometric assay (IRMA) of SHBG is increasingly popular (3). However, techniques with labeled antibodies require sample pretreatment, involve several manual procedures, take at least 2 h to complete, and, for IRMA, necessitate the use of radioisotopes. In contrast, particle-enhanced turbidimetric immunoassays (PETIA) often do not require sample pre-treatment, are readily automated, are completed in <20 min, and avoid the use of radioisotopes (13).

Materials and Methods

Materials

Latex particles. The latex particles, 40 nm in diameter, are based on a polyvinylpyrrolidone core (14) with a chemically reactive shell of chloromethylstyrene (15). A gift from E.I. duPont de Nemours (Wilmington, DE), they were provided as a 100 g/L suspension.

Antibody. We used the immunological fraction of a polyclonal rabbit antiserum to human SHBG, a gift from Dakopatts (Copenhagen, Denmark).

Buffers. All buffers used were at pH 7.5 and contained sodium azide, 0.1 g/L, unless stated otherwise. Antibody was coupled to particles in 15 mmol/L phosphate buffer containing the detergent GAFAC RE610 (GAF, Wythenshawe, Manchester, U.K.), 0.5 g/L. After coupling, particles were washed with 50 mmol/L glycine solution before storage in 200 mmol/L glycine. For use, particles were diluted in 5 mmol/L glycine solution. The buffer used for the assay was 340 mmol/L borate/potassium chloride, pH 8.0, containing polyethylene glycol (PEG) 8000 (Union Carbide, Danbury, CT), 33.8 g/L.

Standards. These were either the standards from a commercially available IRMA kit (Farmos Diagnostica Ltd., Oulunsalo, Finland) with SHBG concentrations between 6.25 and 200 nmol/L or pure SHBG isolated as described previously (16) and diluted in fetal bovine serum (Flow Laboratories, Sydney, Australia). All standards were aliquoted and stored at −20 °C until required. Each aliquot was thawed only once.

Samples

Serum samples were from unselected hospital patients (both sexes) and from women in the third trimester of pregnancy. Serum also was obtained from subjects with increased concentrations of either immunoglobulins or rheumatoid factor. All samples were stored at −20 °C until analyzed.

Apparatus

Turbidimetric reactions were monitored at 340 nm with a Multistat III microcentrifugal analyzer (Instrumentation Laboratory, Lexington, MA). A Cobra Series 5000 Autogamma (Packard Instrument Co., Downers Grove, IL) was used for isotope counting. Electroimmunodiffusion was performed with a flat-bed Multiphor II electrophoresis unit coupled to a Model 2103 power supply (both from LKB Produkter AB, Bromma, Sweden).

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4 Nonstandard abbreviations: FAI, free androgen index; IRMA, immunoradiometric assay; PETIA, particle-enhanced turbidimetric immunoassay; SHBG, sex-hormone-binding globulin; and PEG, polyethylene glycol.

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Procedures

Preparation of antibody-particle reagent. Dialyze particles and antibody, separately, in coupling buffer for 24 h at ambient temperature. Centrifuge the antibody to remove any particulate material and calculate the protein concentration of the supernate from absorbance measurements at 280 nm. In the experiments performed here, we diluted dialedyzed antibody in coupling buffer to give protein concentrations of 1.0, 2.0, and 3.4 g/L. Dilute dialyzed particles 10-fold with coupling buffer to give a 10 g/L suspension before mixing with an equal volume (usually 1.0 or 2.0 mL) of dilute antibody. Incubate the mixture overnight (15–18 h) at 37 °C with continuous shaking, before centrifuging at 40,000 × g for 60 min at 20 °C. Remove the supernate, resuspend the pellet in 4.0 mL of wash buffer, and centrifuge as before. Repeat this procedure four times to ensure removal of any uncoupled antibody. Resuspend the pellet in storage buffer at half the volume of the original mixture of antibody and particles. To ensure total resuspension of the particles, sonicate the mixture at an amplitude of 20 μm for two periods of 30 s. For use, dilute the antibody-particle mixture in 5 mmol/L glycine solution, pH 7.5, to give a suspension with an absorbance in the final reaction mixture of between 0.5 and 0.7 A. Typically this requires a dilution of 25- or 30-fold.

Assays. IRMA was performed with a commercially available method as recommended by the manufacturer (Farmos Diagnostica) and electroimmunodiffusion was carried out as described previously (7).

For PETIA of SHBG, dispense 10 μL of sample or standards, 84 μL of sample-diluting buffer, and 4 μL of distilled water into the inner compartment of the Multistat rotor. Into the outer compartment dispense 200 μL of working antibody-particle reagent and 10 μL of distilled water and incubate the rotor in the instrument until attaining a working temperature of 30 °C. Then mix reactants and read the absorbance at 340 nm 3 s after mixing and again 8 min later. Determine the absorbance change (ΔA) and obtain SHBG concentrations from a manually constructed standard curve. Should sample dilution be necessary, the diluent of choice is sample-diluting buffer (borate/potassium chloride, pH 8.0) without PEG 8000.

Results

Sample-diluting buffer and antisemur concentration. Using antibody-particle reagent prepared with antisera at 3.4 g/L, we analyzed whole fetal bovine serum and a solution containing pure SHBG at about 100 nmol/L in isotonic saline (NaCl, 150 mmol/L), with several buffers with overlapping pH values (pH 6–10). The maximum difference between absorbances of the bovine serum and the SHBG solution was obtained with borate/potassium chloride buffer, pH 8.0. This difference was enhanced by adding PEG 8000 to the borate buffer to give a final PEG concentration of 10 g/L in the reaction mixture. Standard curves for particles coupled with various concentrations of antisemur showed best results with the highest antisemur concentration attainable, 3.4 g/L in this study (Figure 1).

Calibration range. From the value for 3 SD of absorbance measurements of 19 replicates of fetal bovine serum, we determined the lower detection limit of the PETIA to be 2.0 nmol/L. The upper limit of measurement, 320 nmol/L, was determined by measuring increasing concentrations of pure SHBG in fetal bovine serum (Figure 2).

Stability. Stability of this method was assessed by measuring the SHBG concentration of serum standards with the antibody-particle reagent on the day of preparation and after storage in dilute form at 4 °C for four, eight, and 12 weeks. We did not see significant variation in the absorbances of these standards during the assessment period (Figure 3).

Precision. We assessed intra-assay precision by analyzing 17 aliquots from each of three serum pools. These pools contained low (21 nmol/L), intermediate (51 nmol/L), and high (104 nmol/L) concentrations of SHBG; a single rotor was used for each pool. The CVs for these determinations were 4.3%, 1.3%, and 3.0%, respectively. Interassay CVs, determined by analyzing each serum pool on nine occasions during a six-week period, were 7.6%, 4.9%, and 3.9%, respectively. We also analyzed duplicate aliquots of each of the three serum pools twice daily for 20 days (n = 80 for each pool), using separate rotors for each run. We determined the SHBG concentration of these samples both from standard curves prepared daily and from a standard curve prepared on the first day of the 20-day assessment period. From the daily standard curves, mean values (±SD) for the low, intermediate, and high samples were 12.4 (1.0), 56.3 (4.6), and 161.2 (5.6) nmol/L, respectively. Corresponding values from the standard curve prepared on the first day were 12.5 (0.9), 56.3 (3.3), and 159.8 (7.0) nmol/L. From the daily standard curves, the within-day CVs for the low, intermediate, and high serum pools were 4.9%,
2.2%, and 0.8%, respectively. From the standard curve prepared on the first day, these values were 4.0%, 2.1%, and 1.0%. Interassay CVs from the daily standard curves were 8.2%, 8.2%, and 3.5%; from the standard curve prepared on the first day, these were 7.5%, 5.8%, and 4.4% for the low, intermediate, and high pools, respectively.

**Analytical recovery and parallelism.** To assess analytical recovery, we added various volumes of pregnancy serum containing 250 nmol of SHBG per liter to a serum sample containing a low (25 nmol/L) concentration of SHBG. Mean recovery (n = 6) was 101.5% (SD 9.2%), with measurements of SHBG concentration ranging from 39 to 138 nmol/L. We assessed parallelism by serially diluting six serum samples with SHBG concentrations ranging from 130 to 255 nmol/L. Lack of parallelism was not detected from these.

**Comparative measurements.** We determined SHBG concentrations by IRMA and PETIA in serum samples from 119 anonymous hospital patients and in 10 serum samples from women in the third trimester of pregnancy. The SHBG concentrations of these samples ranged from 10 to 471 nmol/L. From regression analysis, PETIA = 0.98 (SD 0.001) × IRMA + 1.69 (SD 0.11) nmol/L, with a correlation coefficient (r) of 0.99 and a standard error of estimate (S_p) of 10.3 nmol/L.

We also made comparative measurements by electroimmunodiffusion to determine the influence of abnormal components on the method. For this we used samples (n = 14) with rheumatoid factor concentrations ranging from 50 to >2500 int. units/mL and samples (n = 5) with paraprotein concentrations of 5 to 35 g/L. (Electroimmunodiffusion is not affected by these abnormal components.) For the samples containing rheumatoid factor, PETIA = 1.05 × EID − 3.45 nmol/L (r = 0.99); and for the paraprotein-containing samples, PETIA = 0.96 × EID − 3.70 nmol/L (r = 0.99). Regression analysis of the combined results gave PETIA = 1.06 (SD 0.01) × EID − 4.5 (SD 0.43) nmol/L (r = 0.99) with an S_p of 4.7 nmol/L (Figure 4).

We also assessed the effects of hemolysis, icterus, and lipemia. Hemoglobin, from lysed erythrocytes, and bilirubin (Sigma Chemical Co.) were added separately to serum to a maximum concentration of 2.0 g/L and 340 μmol/L, respectively. Neither of these compounds at their maximum concentration had a discernible effect on the assay. To assess the effect of lipemia, we added to serum containing triglyceride (9.3 mmol/L) pure SHBG at 22 or 131 nmol/L. Analytical recoveries of the pure protein were not significantly different from those obtained after adding it to samples having normal concentrations of triglyceride.

**Discussion**

PETIA has significant advantages for measuring serum SHBG. The method is simple and rapid and makes use of stable reagents. The upper limit of measurement, ~320 nmol/L, is a concentration rarely reached except in late pregnancy; sample dilution, therefore, is seldom necessary. In contrast, methods currently popular for
measuring serum SHBG require considerable sample dilution. The use of whole rather than diluted serum in PETIA not only reduces technical time but, more importantly, eliminates a potential source of error. Washing and separation steps also are not required for the PETIA, with obvious benefit.

Sample processing involves rotor loading followed by sample analysis. Rotor loading, which is automated, takes about 5 min; sample analysis, including printing of results, requires about 10 min. Each rotor holds 19 samples, so processing of the first 13 samples and six standards takes about 15 min. While processing the samples in one rotor, however, one can be loading other rotors. Therefore, the time taken to process subsequent rotors is effectively only 10 min per rotor. Because analysis of a full set of standards is required at most only once per run, the total time for processing, e.g., 100 samples is about 1 h, far less time than is required to analyze this number of samples by any other assay for SHBG.

Accuracy and precision of the method are virtually identical to those reported recently for immunoassays involving labeled antibodies (11,12), although, as would be expected, sensitivity is less. However, the detection limit of 2.0 nmol/L for the PETIA is about four times lower than the minimum concentration of serum SHBG in males (12). This is sufficiently sensitive for measuring serum concentrations of the protein. Indeed, as Niemi et al. (12) noted recently, extreme levels of sensitivity are not required for this assay. Perhaps the sensitivity of the PETIA would be increased if antibody were coupled to particles at a concentration greater than that used here; however, the antibody concentration we used was the maximum obtainable with the available antiserum.

The performance of the antibody-particle reagent showed little change after storage at 4°C for three months. Similar findings have been reported for antibody-particle reagent stored for 20 months; possible reasons for this stability have been proposed (17). We suppose, therefore, that the antibody-particle reagent used here will have similar stability. No significant differences were seen in mean values or in precision obtained either from standard curves prepared daily during a 20-day assessment period or from a standard curve prepared on the first day of the assessment period. This indicates that full calibration of the method each day is not essential, which reduces both technical time and reagent costs.

Abnormal concentrations of hemoglobin, bilirubin, or triglyceride or the presence of abnormal proteins were found not to affect the method. This is in accord with other assessments of PETIA (13, 17).

Because the samples were from anonymous patients attending various hospital clinics, we could not use these data to construct reference ranges for the method. From the comparisons with the IRMA, however, it is unlikely that the reference ranges for the two methods would differ significantly.

Bordin et al. (9) warned that immunoassays for SHBG should be viewed with caution because results may not reflect the steroid-binding capacity of the protein. This, as yet, has not been proven because several studies have found no differences between results obtained from immunoassays and from steroid-binding assays for SHBG (7,12,18). Therefore, we did not perform comparisons between PETIA and steroid-binding assays.

According to a recent survey, the measurement of serum SHBG is rapidly increasing in popularity, because of both a greater awareness of the role of the protein in androgen-related disorders and the availability of a commercial IRMA (3). It also has been suggested that measurement of the protein may be useful, in conjunction with measurements of other analytes, as a biochemical index of thyroid hormone action in peripheral tissues (19). Presumably, therefore, the routine measurement of serum SHBG will continue to increase. Use of PETIA for measurement of the protein in serum has advantages for the routine laboratory. Our comparisons show that measurement of SHBG in serum by PETIA is simpler and considerably faster than measurement by a commercial IRMA, and that the analytical performance of the two methods is similar. Because centrifugal analyzers are available in most clinical laboratories, the addition of a PETIA for serum SHBG to their repertoire provides an attractive alternative to existing methods for its measurement.

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