Immunoassay for Sex Hormone-Binding Globulin in Undiluted Serum Is Influenced by High-Molecular-Mass Aggregates

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Background: The new Elecsys® chemiluminescence assay for measurement of homodimeric sex hormone-binding globulin (SHBG) was designed for use with undiluted serum, in contrast to other methods that require predilution. During assay development, unexpected calibration difficulties were observed that were attributable to particular biochemical properties of the highly concentrated SHBG in solution.

Methods: We used a surface plasmon resonance (SPR) biosensor, which enables biomolecular interaction analysis of SHBG, and size-exclusion chromatography for this investigation. The immunoassay was evaluated for imprecision, linearity, and suitability of the dilution medium, and the method was compared with an IRMA for SHBG.

Results: The SPR biosensor characterized the special protein properties of SHBG in various concentrations. Above 200 nmol/L there was a strong tendency toward formation of high-molecular-mass aggregates. This was also detectable by size-exclusion chromatography and could be reversed by simple dilution of the sample. On the basis of these results, the dynamic measuring range of the SHBG assay is restricted to 0.350–200 nmol/L. Assay evaluation on a 2010 analyzer revealed excellent precision (CV <2.5%). Mean recoveries were 84.2–98.8%. Intermethod comparison with an IRMA yielded a satisfactory concordance of the two assays with a Spearman correlation coefficient of 0.8807.

Conclusions: Aggregates of human SHBG may have a detrimental impact on the accurate measurement of the protein if measurements are performed with undiluted serum samples. Further work is needed to clarify whether these high-molecular-mass aggregates influence the free fraction of steroid hormones in vivo.

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Human sex hormone-binding globulin (SHBG), a 93.4-kDa homodimeric glycoprotein produced by hepatocytes, is the major sex steroid-binding protein in plasma. This transport protein binds testosterone, 5α-dihydrotestosterone (DHT), and 17β-estradiol in plasma with high affinity (1–4). Its known physiologic roles include the regulation of bioavailability of testosterone and 17β-estradiol via control of their respective metabolic clearance rates (5–8); roles for SHBG in the cellular uptake of sex steroids (9–11) and in signaling (12, 13) have also been proposed. Plasma SHBG concentrations vary considerably among individuals and are influenced by hormonal, metabolic, and nutritional factors. It is of clinical interest that serum SHBG concentrations are low in women suffering from disorders characterized by androgen excess (14), as well as in patients of either sex with hyperinsulinemia or hypothyreosis. The androgen excess is usually quantified by direct measurement of free testosterone or by the free androgen index, calculated by the ratio of the measured concentrations of total testosterone and SHBG. SHBG is increased in pregnancy and in women taking synthetic estrogens, as well as in patients suffering from hyperthy-
roidism (14). Clinical studies have shown that measurement of SHBG in serum is an important additional indicator for monitoring of thyroid function; the diagnostic value is, however, limited to hyperthyroidism (15). Moreover, low SHBG could be associated with an increased risk for developing type II diabetes and cardiovascular diseases. Low SHBG concentrations have also been observed to be strongly linked to the metabolic syndrome (16).

The electrochemiluminescence (ECL) immunoassay technique from Roche Diagnostics (performed on the automated analyzer platforms Elecsys® 1010, 2010, and E170) is based on the electrochemically generated chemiluminescent reaction by a ruthenium complex (17, 18). Recently, the Elecsys SHBG assay for in vitro quantitative measurement of SHBG in human serum and plasma was developed. Surprisingly, during development of the assay, unexpected difficulties were found in calibrating the assay for use with undiluted serum samples. The speculation of unusual protein properties led us investigate the above-mentioned problems, using a biosensor. We used a biosensor device with a photometric transducer, based on the surface plasmon resonance (SPR) technique. The sensor detects changes in mass concentrations in the surface layer during the course of a biospecific interaction event as changes in the refractive index via the SPR effect (19, 20). This optical phenomenon produces a reduction of the light intensity reflected over a thin gold film interfacing the glass support and the surface of a sensor chip. The effect is maximum at a particular (resonant) angle, which varies as a function of the refractive index according to the changes in mass concentration of the soluble protein interacting with ligands covalently attached to the flexible carboxymethyl-dextran matrix of the sensor chip. Generally, the interaction of proteins/receptors with their respective ligands, immobilized on the sensor chip, is monitored as a function of time (as SPR sensograms), expressed in arbitrary resonance units (RU). One RU represents a change of 0.0001 degree in the angle of the intensity minimum. For most proteins (including SHBG), this is roughly equivalent to a change in concentration of ~1 pg/mm² on the sensor surface. As described recently (21), we investigated with this technique the biospecific interaction of SHBG with several immobilized androgenic ligands and found in a global fitting analysis of the biosensor data that the interaction of steroids with SHBG was best described by a bivalent kinetic model. These measurements were, however, valid only when SHBG in solution was added in concentrations <200 nmol/L. Above this limit, the kinetic analysis was presumably corrupted by a second protein species in the sample. At that time, the presumption was that a SHBG aggregate might be the second species.

Here we describe unexpected difficulties in finding a calibration procedure for the assay for use with undiluted serum samples; these problems resulted from particular biochemical properties of highly concentrated SHBG in solution. We also present evidence of aggregated SHBG forms as detected by SPR biosensor measurements and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Additionally, we present the data from a first evaluation of the ECL immunoassay.

**Materials and Methods**

### Study Samples

We investigated 168 routine sera from adult patients of either gender in equal numbers, presenting with various diseases; 7 of the women were pregnant (second trimester). Exclusion criteria were disorders of lipoprotein metabolism and multiple myeloma with increased monoclonal immunoglobulins. Sera not being analyzed immediately were stored at −20 °C before analysis.

### Instruments, Reagents, and Procedures

#### Instruments

We evaluated the SHBG assay on an Elecsys 2010 automated analyzer. The SPR biosensor Biacore X® (Biacore AB) was used for ligand-binding measurements. The interaction of the transport protein with its ligands, immobilized on a Pioneer B1 chip, was monitored over time and reported in sensorgrams (RU vs time). For size-exclusion chromatography, we used an AKTA FPLC system from Amersham Pharmacia. We applied 0.1 mL of each sample to a Superdex (composite matrix of dextran and agarose) 200 HR 10/30 gel filtration column (10 × 300 mm) equilibrated with phosphate-buffered saline and run at 0.2 mL/min. The column effluent was monitored by ultraviolet detection at 280 nm, and 0.25-mL fractions were collected. Gel electrophoreses (SDS-PAGE) and protein transfer were performed with the MiniProtean 3 cell system (Bio-Rad). Immunoblotting was performed as described previously (21). Immunoreactive bands were resolved with use of the SuperSignal® enhanced chemiluminescence reagent (Perbio Science) as substrate for horseradish peroxidase conjugated to a goat anti-rabbit antibody, and Hyperfilm™ (both from Amersham).

#### Chemicals

The polyclonal rabbit anti-SHBG antibody for immunoblotting was from Dako A/S. Traces of contaminating antibodies were removed by solid-phase extraction with human plasma proteins. For the biosensor experiments, and partly for size-exclusion chromatography, we used affinity-purified SHBG from human pregnancy plasma (>98% by SDS-PAGE) purchased from Fitzgerald Industries International. The lyophilized powder was reconstituted in doubly distilled water at a concentration of 5 g/L. B1 sensor chips (with a stable carboxymethyl-dextran matrix, characterized by a low degree of carboxylation) and HEPES-buffered saline were purchased from Biacore. All other chemicals were from Sigma.

#### Immunoassay Tests

The Elecsys SHBG immunoassay uses two monoclonal anti-human SHBG antibodies. The SHBG CalSet consists of lyophilized equine (Cal1) and human (Cal2) sera. The assay has a measuring range of 0.350–200 nmol/L. Cal2) sera. The assay has a measuring range of 0.350–200 nmol/L.
nmol/L and is standardized against the 1st International Standard for SHBG from the National Institute for Biological Standards and Control (code 95/560). For the intermethod comparison, we used a SHBG IRMA from Orion. The IRMA method, extensively described by Hammond et al. (22), is standardized against a discrete determinant on the SHBG molecule. For measurements, patient sera, calibrators, and controls are diluted 1:100 with assay buffer. The lowest detectable concentration, as given by the company, is 0.5 nmol/L. The total measurement range is 6.25–200 nmol/L, whereas the linear measuring range is 6.25–100 nmol/L, with intraassay CVs of 2–5%.

**SPR biosensor measurements.** The chip surface in this study was produced by covalent attachment of the androgenic compound 1α-amino-hexyl-17β-hydroxy-5α-androstane-3-one (23) to the carboxymethyl-dextran matrix of a B1 chip, as described in detail by Metzger et al. (21). The flow cell (FC2) of the biosensor instrument was used for coating the steroidal ligand, whereas the reference cell (FC1) was used for a similar coating in the absence of steroid to allow subtraction of nonspecific binding phenomena (ΔRU). For measurements, we chose a temperature of 25 °C and a flow rate of 10 μL/min. We applied SHBG in various concentrations (final concentrations, 12.5–800 nmol/L) over the solid-state surface. This is the association phase, its endpoint being defined as $R_{eq}$ (relative binding capacity in ΔRU near steady state). Thus, $R_{eq}$ reflects the optimum association of SHBG to the immobilized 1α-aminoalkyl-DHT. After this, the switch to buffer injection started the dissociation phase. For regeneration, 100 mmol/L H$_3$PO$_4$ was added at the end of the delay time (600 s), marking the dissociation phase.

**Evaluation protocol.** We assessed the Elecsys assay performance characteristics in an α-site study, based on the ECCLS guidelines (24), with the following evaluation features: For intraassay imprecision, we measured 21 aliquots of each of the Elecsys PreciControl Universal 1 and 2 controls in one run. For the interassay imprecision, we measured the SHBG concentrations in the two controls and six serum (pool) samples in 10 separate runs with 2 runs per day. We assessed assay linearity with three serum samples with SHBG concentrations near the upper end of the measuring range; each was serially diluted with a lower-concentration serum sample. We determined the ratio of the measured vs the expected SHBG concentration in nine different dilutions. All dilutions were assayed in duplicate, whereas the undiluted serum and the low-concentration samples were assayed six times. We evaluated the suitability of the dilution medium by diluting high-concentration sera with the Elecsys Diluent Universal (matrix consisting of phosphate-buffered saline containing 10 g/L bovine serum albumin) instead of a low-concentration serum sample. For the intermethod comparison, we assayed 168 sera from single donors covering the entire analytical range in singlet with the ECL assay and the Orion IRMA. In case of obvious outliers, the respective sample was reanalyzed with both methods. The repetition value replaced the previous result if accordance could be reached. In case of persisting discrepancies, the re-run in both methods was taken as the overall result.

**Statistics.** Statistical parameters were calculated by use of add-in macros in Windows Excel®. The intermethod comparison was performed with the nonparametric regression analysis of Passing and Bablok (25), and Spearman correlation coefficients were calculated.

**Results and Discussion**

**ECL assay calibration**

The two-point recalibrations for the ECL assay, intended for use with undiluted serum, unexpectedly failed when purified SHBG was added to a serum-based calibrator matrix. It was impossible to obtain linearity when higher-titer native human sera were diluted with low-concentration samples or with Elecsys Diluent Universal. As a consequence, calibrators were developed based on native human serum samples with different endogenous SHBG concentrations. Seven master calibrators (0, 5, 13, 28, 73, 151, and 208 nmol/L) were then standardized against the National Institute for Biological Standards and Control standard code 95/560.

**Detection of aggregated SHBG**

The original intent of the assay was for analysis of undiluted serum samples, which is in contrast to all other SHBG immunoassays on the market, which typically require 1:100 dilution of serum. The described problems in establishing SHBG calibrators for the Elecsys assay, however, led us to investigate the particular biochemical properties of high concentrations of SHBG in solution. We used SHBG that was affinity-purified from human pregnancy plasmas. The stability and binding activity were assessed as described previously (21, 23). To improve homogeneity of the SHBG preparation used for biosensor experiments, we deglycosylated the N-linked carbohydrates with PNGase F, as described elsewhere (21). The functional binding activity of the product was found to be unaffected (21).

The following experimental observation gave important information in solving the problem of the calibrators for the Elecsys assay: The SPR binding studies of the interaction of immobilized 1α-aminoalkyl derivatives of DHT with the purified N-deglycosylated homodimeric human SHBG in solution indicated that reproducible results were obtained only in a concentration range of 10–200 nmol/L. Above this concentration, ambiguous data were obtained during the curve fitting analysis. As shown in Fig. 1A, these binding curves (400 and 800 nmol/L) still have the same shape as those for curves at concentrations <200 nmol/L. The relationship of the ΔRU values at $R_{eq}$ with the respective SHBG concentrations
showed, however, that the slope of the hyperbolic saturation curve was abruptly attenuated at concentrations above 200 nmol/L (Fig. 1B). This observation may be explained by the presence of a second protein species, presumably a SHBG aggregate, in the solution. An artifact caused by an excessively high ratio of SHBG to the immobilized ligand can be ruled out by carefully conducted kinetic analysis of each sensorgram, which would clearly indicate putative saturation.

Subsequently, we performed analytical size-exclusion chromatography of the affinity-purified N-deglycosylated SHBG preparation at a concentration of 11 μmol/L at 25 °C according to a protocol described elsewhere (23). The chromatogram (Fig. 2A) showed several peaks in addition to the expected SHBG main peak at 62–65 min, although the protein was highly pure. These peaks were found at retention times of 39–40 and 53–56 min and resembled the peaks present in a chromatogram of affinity-purified SHBG that was notPNGase F digested (Fig. 2B). The respective SHBG peaks in Fig. 2B were visualized by Western blotting using polyclonal anti-SHBG and horseradish peroxidase-conjugated goat anti-rabbit antibody. Signals attributable to proteins cross-reactive with the antibody could be excluded because the high specificity of the anti-SHBG antibody had been verified by crossed immunoelectrophoresis by the manufacturer. Moreover, we found no additional immunoreactive bands in several serum samples with high and low SHBG concentrations as well as by use of plasminogen and protein S solutions in immunoblotting experiments. We performed an analysis of the testosterone-binding activity of the respective size-exclusion chromatography fractions (23) and observed significant binding of testosterone in the fraction with the aggregates (data not shown). We checked the temperature dependence of the observed aggregate formation and found similar immunoblot signals at 4 and 37 °C (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue2/).

We also performed size-exclusion chromatography experiments with individual sera from pregnant women to confirm that the findings with purified SHBG were not attributable to artifacts of the purification procedure. Four sera with endogenous SHBG concentrations of 300–400 nmol/L were gel-chromatographed at 25 °C, and the respective SHBG peaks were visualized by Western blotting and immunoassay detection of SHBG in the individual fractions. As shown in Fig. 3 for one representative individual serum, the broad peak in the high-molecular-mass range (∼1000 kDa) in addition to the characteristic peak of the homodimeric protein indicates the presence of high-molecular-mass aggregates. ECL measurements of SHBG in the respective chromatography fractions confirmed the additional peak.

We also serially diluted four individual sera from pregnant women with high endogenous SHBG concentrations (>400 nmol/L). The recovery plot of the ECL assay (Fig. 4) indicated that the ECL signals did not change in a linear manner when the dilution factor was 1 or lower. The corresponding borderline concentrations of SHBG at ECL signals of 1.0–1.2 × 10^6 were in fact >200 nmol/L. Because the Elecsys assay is designed for use with undiluted serum, measurement of higher-concentration samples (2 dilution and undiluted) of the four respective sera gave falsely low SHBG concentrations as a result of aggregates with altered immunogenicity. The signals of all measured samples with SHBG concentrations <200 nmol/L were in close correlation to the signals for the seven calibrators and to the signal for the recalibration at 36.40 nmol/L. These measurements support the hypothesis of oligomerization of the glycoprotein.

**ASSAY PERFORMANCE DATA**

The intraassay CVs were 1.1% for the low control and 1.7% for the high control. The interassay CVs were 1.3–2.5%, depending on the sample being used. All results are summarized in Table 1 of the online Data Supplement.
The three serum samples used for linearity testing gave SHBG concentrations of 182.1 nmol/L (sample 1), 176.7 nmol/L (sample 2), and 161.8 nmol/L (sample 3). The three samples were diluted with sera containing low endogenous SHBG concentrations (sample 1, 16.3 nmol/L; sample 2, 16.5 nmol/L; sample 3, 16.8 nmol/L). The measured recoveries were 89.3–99.7% (mean, 94.4%) with better values for lower dilutions. When we diluted the same sera with the Elecsys Diluent Universal, the observed recoveries were 84.2–98.8% (mean, 91.4%). In this dilution series, we found pronounced discrepancies in the more highly diluted samples. The results for the dilutions with sera containing low endogenous SHBG concentrations, as well as with Diluent Universal, fell within the recommended range of ±15% (Fig. 5). A trend showing a lack of recovery at increasing dilutions was observed for the Diluent Universal dilution.

We compared the results obtained with the Elecsys assay with those obtained with the Orion IRMA. For 168
examined undiluted serum samples, linear regression analysis according to Passing and Bablok yielded the linear regression equation: $y = 1.1494x + 3.5345$ (Spearman correlation coefficient $0.8807$). The respective experimental data are given in the online Data Supplement.

The present investigation demonstrates that human SHBG forms in vitro aggregates such as oligomers at higher concentrations. This could be shown both in samples of purified SHBG and in sera with high endogenous concentrations (>200 nmol/L). Binding to other proteins in the serum samples, however, cannot be excluded. There are many descriptions of specific oligomerizations of a series of proteins in the literature, but the only description of aggregate-forming properties of serum proteins was given by Jensen et al. (26). These authors observed polymerization of hemopexin and haptoglobin during heating of a human plasma albumin solution to 60 °C.

Our results indicate a potentially detrimental impact on the accurate measurement of SHBG in serum by immunoassays if serum samples are used without dilution: Concentrations in samples with high SHBG would be possibly underestimated. This analytical problem of SHBG aggregates is not relevant, however, for immunoassays that use a 1:100 serum dilution before determination. Nevertheless, SHBG measurements by automated immunoassay systems are more easily performed without a pretest dilution step.

The analytical problem became evident during the efforts to standardize the Elecsys ECL assay. The upper limit of the measuring range of the SHBG assay had to be restricted to 200 nmol/L. The problem observed in assay development was solved by use of calibrators prepared from native human serum samples. These had individual endogenous SHBG concentrations, and no SHBG was added to these calibrators.

The crucial experimental observation that led to the hypothesis of the aggregate-forming property of SHBG was obtained by SPR biosensor experiments initially performed to characterize the biomolecular interaction of SHBG to androgenic ligands (21). As shown in Fig. 1, the SPR biosensor clearly unmasks heterogeneous interactions at higher concentrations and thus gives an indication of SHBG aggregates. The high-molecular-mass aggregate peak was detectable at 4 and 37 °C. More biochemical investigations are needed to better understand the exact protein chemical nature of the aggregate-forming process in relation to temperature and pH.

As a consequence of this analytical issue, the SHBG ECL assay (launched in mid-2003) was restricted to the dynamic range of 0.350–200 nmol/L.

In conclusion, SPR biosensor and gel chromatography investigations indicated that SHBG forms high-molecular-mass aggregates. Further work is needed to clarify whether these protein aggregates are also present in vivo. One can speculate that such a finding would shed new light on the bioavailability of estrogens in pregnancy and in other clinical situations with increased serum SHBG.

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References

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