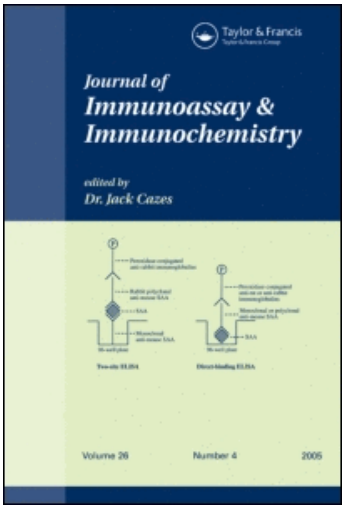

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Two-Sites Immunoradiometric Assay Using Monoclonal Antibodies for the Determination of Serum Human Sex Hormone Binding Globulin

Claude Cox[a]; Cécile Caulier[a]; Guy Havelange[a]; Jean Claude Meunier[b]; Antonella Renzi[c]
[a] TECHLAND S.A., Parc Industriel du Sart-Tilman, Angleur-Liège, Belgium [b] Hôpital Civil de Charleroi, Laboratoire de Biologie Clinique Boulevard, Janson, Charleroi, Belgium [c] RADIM s.p.a., Roma, Italy

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TWO-SITES IMMUNORADIOMETRIC ASSAY USING MONOCLONAL ANTIBODIES FOR THE DETERMINATION OF SERUM HUMAN SEX HORMONE BINDING GLOBULIN.

Claude Cox [1], Cécile Caulier [1], Guy Havelange [1], Jean Claude Meunier [2], and Antonella Renzi [3].
[1] TECHLAND S.A., Parc Industriel du Sart-Tilman,
4031 Angleur - Liège Belgium
[2] Hôpital Civil de Charleroi, Laboratoire de Biologie Clinique
Boulevard Janson,92
6000 Charleroi Belgium
[3] RADIM s.p.a.,Via del Mare,125,
00040 Pomezia - Roma Italy

ABSTRACT

An immunoradiometric assay (IRMA) for the determination of human Sex Hormone Binding Globulin (SHBG) in serum is described. This IRMA uses three mouse monoclonal antibodies.
Two monoclonals anti-human SHBG are coated on tubes and used as capture antibodies. The third monoclonal labeled with ^{125}I completes the system, allowing the formation of the "sandwich".
The detection limit of the assay is 2.5 femtomol SHBG per tube (250 pg/tube).
Using this test for the measurement of SHBG and radioimmunoassays for the determination of total Testosterone and Estradiol, we calculated the Free Androgen Index (FAI) and the Free Testosterone. The results obtained were compared with the values of Free Testosterone measured by equilibrium dialysis.
There is a close correlation between both calculated parameters and the levels of Free Testosterone, validating this SHBG assay.

(KEY WORDS : sex hormone binding globulin, monoclonal antibodies, immunoradiometric assay, free androgen index, free testosterone).

INTRODUCTION

Sex Hormone Binding Globulin (SHBG) also known as Testosterone-estradiol Binding Globulin (TeBG) is a circulating glycoprotein with a molecular weight of about 90 kDa and is constituted of two different protomers (1).

The protein has one binding site characterized by a very high affinity for steroidal sex hormones such Estradiol, Testosterone and 5Alpha-Dihydrotestosterone.

The most important role of SHBG is as a transport protein for estrogens and androgens in the peripheral circulation. Binding to SHBG reduces the metabolic degradation of these steroids and the high affinity will also affect and regulate the relationship between the amount of hormone which is free and bound. SHBG regulates the free form of sex steroid hormones which is generally assumed to be the main biologically active form in plasma (2).

Measurement of SHBG can also be used for calculation of free androgens and estrogens levels in serum (3, 4).

Several methods have been described to measure serum SHBG. The first described methods were direct binding assays using tritiated 5Alpha-Dihydrotestosterone as ligand (5, 6). A liquid phase immunoradiometric assay has also been described using monoclonal and polyclonal antibodies (7). Some RIA's for SHBG have also been used (8, 9).

The aim of our study was to develop a coated tube immunoradiometric assay for human SHBG using monoclonal antibodies and to validate this assay by comparison of the calculated Free Androgen Index

and the calculated Free Testosterone with Free Testosterone levels measured by equilibrium dialysis in normal and hirsute women.

MATERIAL AND METHODS

Reagents

Protein A-Sepharose, Con A-Sepharose and Superose 12 were purchased from Pharmacia (Uppsala, Sweden). Maxisorp tubes were purchased from Nunc (Roskilde, Denmark). Iodo-Gen was obtained from Pierce (Rockford, USA). $Na^{125}I$ carrier free and tritiated Testosterone were products from Amersham (Little Chalfont, England). Horse serum was a product from Gibco (Gent, Belgium). All other reagents were of analytical grade.

Purification of human SHBG

Human SHBG was isolated from third trimester pregnancy serum by affinity chromatography as previously described (10).

The protein was further purified by adsorption on Con A-Sepharose and eluted with Alpha-Methyl-D-Mannoside.

After concentration, it was further chromatographied on Superose 12 and the second peak eluted was identified as pure human SHBG. The solution was concentrated to approximately 1 mg/ml and stored frozen.

The product was found homogeneous in SDS-PAGE giving a molecular weight of about 90 kDa.

^{125}I labeled SHBG was prepared by iodination of the purified SHBG using Iodo-Gen according to the instructions of the manufacturer. The ^{125}I-SHBG had a specific activity of 20 mCi/mg.

Production of monoclonal antibodies to SHBG

Seven weeks-old female Balb/c mice (Charles River) were immunized with purified SHBG in complete Freund's adjuvant (15 μg/mouse) and boosted twice at monthly intervals by intra-peritoneal injections. After two months every mice showed serum anti-SHBG antibodies and one of them with highest titer was selected and received further 50 μg antigen intravenously, three days prior to fusion.

Immuno spleen cells were hybridized with non-secreting mouse myeloma in the presence of polyethylene glycol by the hybridoma technique of Kohler and Milstein (11). Fused cells were seeded in 24-wells plates (Nunc,Roskilde,Denmark) with Iscove modified Dulbecco's medium (Gibco,Gent,Belgium) supplemented with 2 % hypoxanthine / thymidine / aminopterin and Balb/c mouse peritoneal macrophages as feeders.

The culture supernates of the growing hybrid cells were screened for anti-SHBG activity after 14 days using a liquid phase RIA procedure with ^{125}I labeled SHBG prepared as above. We obtained a panel of sixteen positives which were able to bind ^{125}I-SHBG. Five of them with a binding ranging from 18 to 26 % of the total activity were cultured and cloned at least twice by limiting dilution.

The immunoglobulin subclasses of the five selected monoclonals denoted C12, 8D6, C5, 12B6, F6 were of the IgG1 and IgG2a isotypes as determined by the Ouchterlony's immunodiffusion technique.

Their affinity constants ranged from 10^9 to 10^{10} l/mol as estimated by a modified Scatchard method (12).

The cloned hybridomas were propagated as ascites in Freund's incomplete adjuvant primed Balb/c mice (20 g weight). Ascitic fluids were collected 7 to 15 days after inoculation and stored at -20°C.

Coating of the antibodies

The antibodies produced in ascitic fluids were purified on Protein A-Sepharose according to the instructions of the manufacturer. The purified IgG were concentrated to 1 mg/ml in phosphate buffer and kept frozen.

Antibodies were coated in Maxisorp tubes at a concentration of 10 μg/ml in carbonate/bicarbonate buffer, pH=9.2 during 18 hours at room temperature (300 μl/tube). The solution was discarded and the tubes were saturated with phosphate buffer, pH=7.4 containing BSA 5 g/l, during three hours. After that, the tubes were washed three times with the same solution and dried under vacuum. They were stored at 4°C with dessicant.

Labeling of the antibodies with 125I

IgG purified on Protein A-Sepharose as described above (50 μg) were labeled with ^{125}I using the Iodo-Gen method at a specific activity of 10 mCi/mg. Purification of the labeled antibodies was performed by gel filtration on Sephadex G25. After purification, labeled antibodies were diluted at approximately 100,000 cpm per 200 μl in phosphate buffer, pH=7.4 containing BSA 5 g/l and stored at 4°C. Mouse IgG were added at a final concentration of 50 μg/ml to avoid interferences from heterophilic antibodies eventually present in the assayed samples.

Human SHBG standard curve

The standard curve was prepared by adding purified SHBG to horse serum. Exact concentrations of the standards were determined using tritiated 5Alpha-Dihydrotestosterone as ligand (5). Concentrations of the standards ranged from 10 to 250 nmol/l.

The standards were further diluted 1:51 in phosphate buffer, pH=7.4 containing BSA 5 g/l.

Immunoradiometric assay for human SHBG

Before assay, samples were diluted 1:51 in phosphate buffer, pH=7.4 containing BSA 5 g/l.

Fifty μl of standards or diluted samples were pipetted in coated tubes and 200 μl of labeled antibody were added. Incubation proceeded for 90 minutes at room temperature on an orbital shaker (Radim, Pomezia, Italy) set at 150 rpm or for two hours at 37°C in a water-bath without agitation.

After incubation, the contents of the tubes was aspirated and the tubes were washed with two ml of 0.05 M Tris buffer, pH=7.4 containing Tween 20 0.1 %.

Radioactivity was measured in a gamma counter (LKB, Broma, Sweden).

RESULTS

Selection of the match pairs of monoclonal antibodies

After selection of the five clones showing the highest affinity for SHBG, each monoclonal has been coated as described above and tested with each of the remaining monoclonals labeled with ^{125}I in order to detect the pairs of monoclonals giving suitable sensitivity in a two-sites immunometric assay. For these tests, we used only 25 μl of standard diluted 1:51 and 200 μl of tracer (100,000 cpm).

The results obtained are summarized in Table 1. The percentage of fixation of the tracer (B/T %) obtained with the standard at 250 nmol/l has only been considered.

TABLE 1

Fixation (B/T %) with each Pair of Monoclonals obtained with the 250 nmol/l Standard.

^{125}I labeled Mab

Coated Mab	12B6	8D6	C12	C5	F6
12B6	----	< 7 %	10 %	< 7 %	< 7 %
8D6	45 %	----	< 7 %	< 7 %	27 %
C12	24 %	< 7 %	----	< 7 %	18 %
C5	40 %	< 7 %	< 7 %	----	38 %
F6	< 7 %	< 7 %	7 %	< 7 %	----

Best sensitivity was obtained with monoclonal 12B6 as tracer and 8D6 as coated antibody. With the same tracer, C5 gave also interesting results.

12B6, C5 and 8D6 were further studied in order to assess if they were directed against distinct epitopes of the SHBG.

For this purpose, ^{125}I labeled SHBG was allowed to bind to the coated antibody, either C5 or 8D6, with increasing amounts of the other monoclonal as competitor.

In this experiment, C5 and 8D6 were found able to inhibit the binding of ^{125}I-SHBG to the other monoclonal, suggesting that C5 and 8D6 were directed against the same epitope or against close epitopes of the SHBG.

On the other hand, 12B6 was unable to displace 3C5 and 8B6 or to be displaced by 3C5 and 12B6, confirming that the monoclonal used as tracer (^{125}I labeled 12B6) was directed against a different epitope of the SHBG than the capture antibodies (C5 and 8D6).

However, in another experiment, we compared the values of SHBG obtained with samples using tubes coated with each separate antibody (C5 or 8D6) or with both antibodies (C5 + 8D6) and 12B6 as tracer.In this case, we found significant differences for some samples which gave lower SHBG values (up to 20 %) when measured with tubes coated with only one monoclonal as compared to the values obtained with tubes coated with both antibodies. So we decided to use an egal mixture of these antibodies C5 and 8D6 as capture antibodies and 12B6 as tracer in the immunoradiometric assay.

Characteristics of the assay

A typical standard curve is represented on Figure 1. The percentage of binding of the tracer ^{125}I-12B6 is reported as a function of the concentration in SHBG in the standard before the 1/51 pre-dilution. The detection limit has been determined at 3 standard deviations from the zero standard. We found a detection limit of 2.5 nmol/l in the sample, that is 50 picomol/l in the pre-diluted sample or 2.5 femtomol per tube.

The assay has been examined for a possible so called high dose hook effect : sera with normal SHBG levels were spiked with purified SHBG up to 1,000 nmol/l, which is much higher than the highest values found in

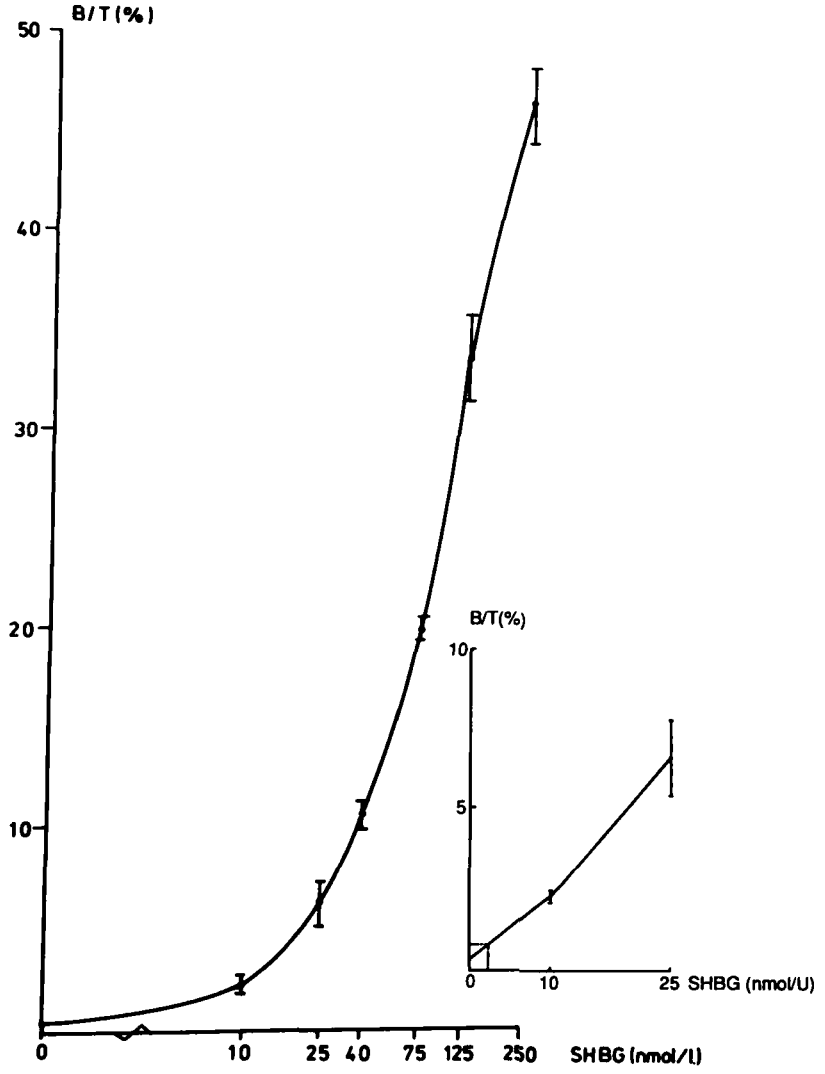

FIGURE 1. Typical standard curve for SHBG. The percentage of the binding of tracer (B/T %) is reported as a function of the concentration of SHBG in the standards (concentration given before the 1/51 pre-dilution). The detailled figure demonstrates the sensitivity of the assay.

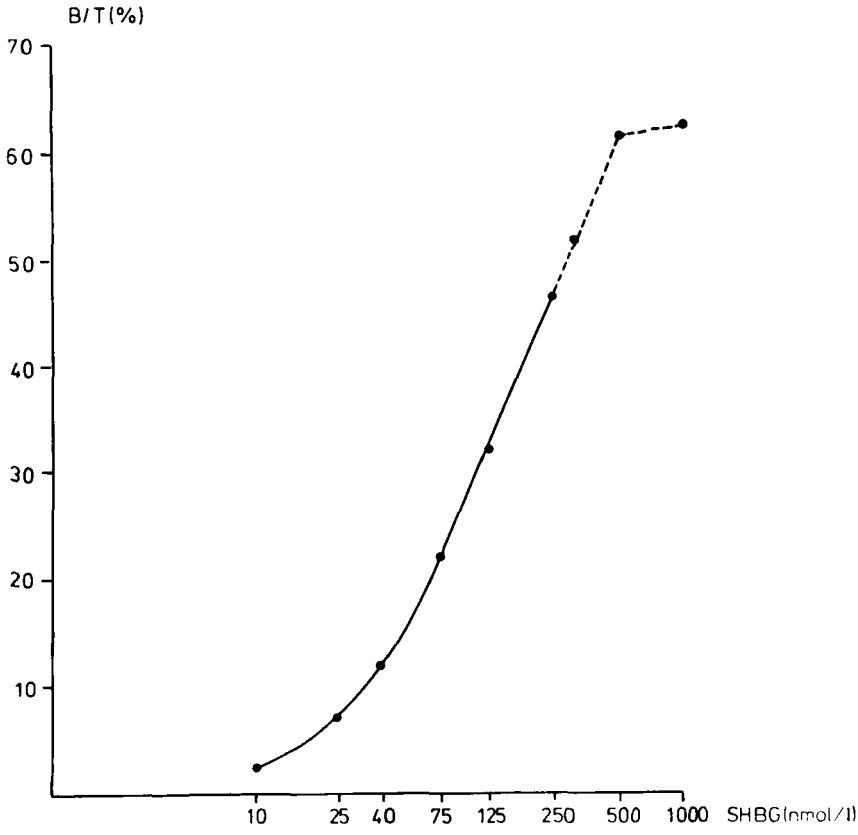

FIGURE 2. Calibration curve with extended range up to 1,000 nmol/l showing no "hook effect".

physiological conditions. All the spiked samples gave signals higher than the last standard as shown in Figure 2 with an extended range calibration curve.

Intra-assay and inter-assay reproductibility has been tested at different levels of SHBG and the results are summarized in Table 2 and 3.

No cross reactions have been found with other human proteins such Thyroxine Binding Globulin, Corticosteroid Binding Globulin, Albumin, Thyroglobulin, Transferrin, Immunoglobulins.

TABLE 2

Intra-assay precision.

N	MEAN	STANDARD DEVIATION	C.V. (%)
10	28.9	1.2	4.2
10	102.3	3.4	3.3
10	198.8	9.5	4.9

TABLE 3

Inter-assay precision.

N	MEAN	STANDARD DEVIATION	C.V. (%)
10	23.4	1.3	5.6
10	65.7	3.0	4.6
10	166.4	8.5	5.1

The accuracy of the method has been evaluated by parallelism and recovery studies.

For the parallelism, samples were assayed both undiluted and diluted with the zero standard. Dilution curves indicating good parallelism between the standard curve and the dilutions of samples are represented on Figure 3.

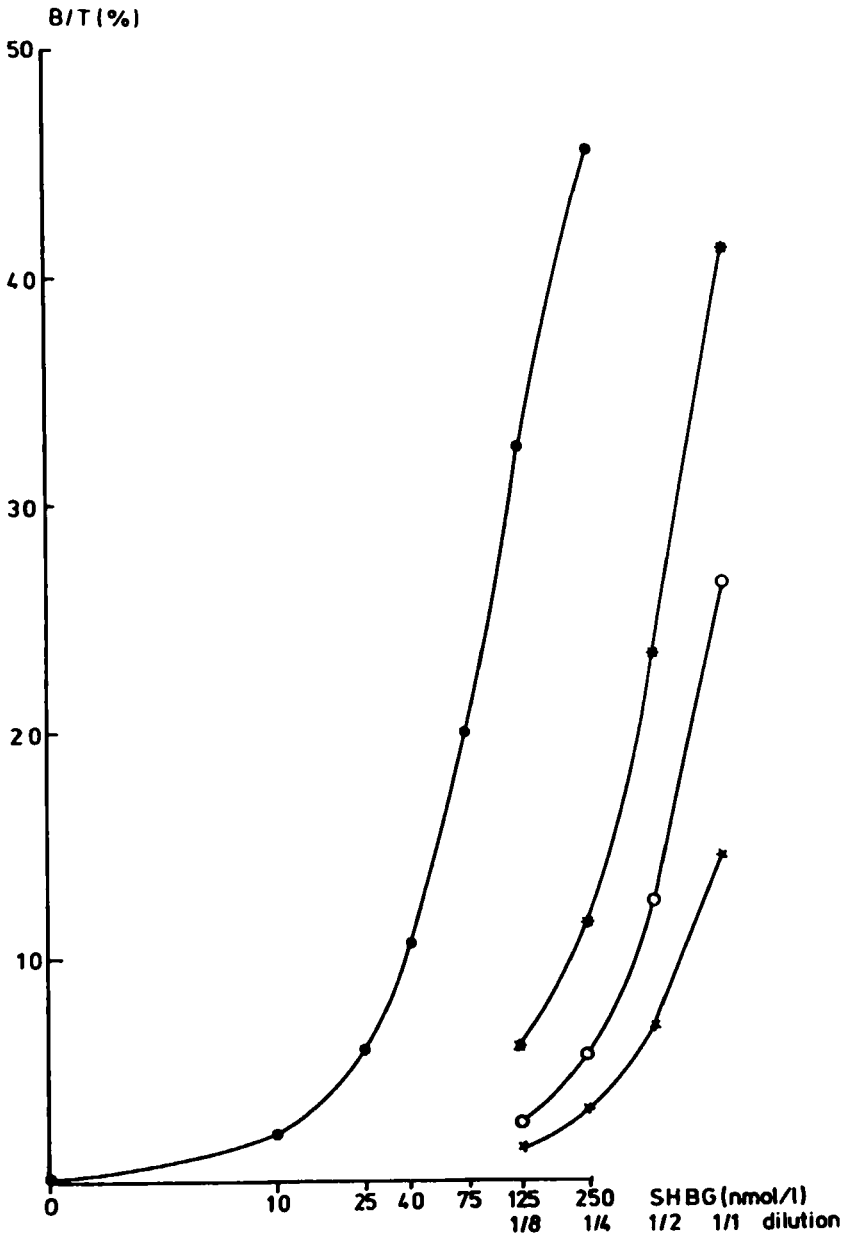

FIGURE 3. Parallelism between the standard curve (●) and dilutions of serum samples (● , o , ★) in the Zero standard.

TABLE 4

Recovery experiment

SAMPLES	OBSERVED	EXPECTED	RECOVERY
1	27.0		
1 + 10	19.3	18.5	104 %
1 + 25	27.3	26.0	104 %
1 + 40	33.2	33.5	99 %
1 + 75	51.5	51.0	101 %
1 + 125	81.4	76.0	107 %
1 + 250	153.0	138.5	110 %
2	53.3		
2 + 10	32.8	31.7	103 %
2 + 25	39.2	39.2	100 %
2 + 40	50.3	46.7	113 %
2 + 75	65.3	64.2	102 %
2 + 125	88.1	89.2	99 %
2 + 250	153.8	151.7	101 %
3	61.0		
3 + 10	34.8	35.5	98 %
3 + 25	41.7	43.0	97 %
3 + 40	49.6	50.5	98 %
3 + 75	63.3	68.0	92 %
3 + 125	88.7	93.0	95 %
3 + 250	148.1	155.5	95 %

For recovery tests, samples were processed in assays which included one to one dilutions of the samples with each of the standards. The percentage of recovery were calculated and are summarized in Table 4.

This assay (IRMA) has been correlated with two commercially available assays : a liquid-phase immunoradiometric assay from Farmos (Oulunsalo,Finland) and a direct RIA from Techland (Liege,Belgium). The

Farmos assay is an immunoradiometric assay with a 1/101 pre-dilution of the sample while the kit from Techland is a direct radioimmunoassay without pre-dilution of the sample. The equations of the regression lines are :

Farmos = 0.939 x IRMA + 11.4	r = 0.9388	N = 98
RIA Techland = 0.984 x IRMA + 1.6	r = 0.9366	N = 98

Evaluation of free testosterone with this IRMA for SHBG

Serum SHBG, Total Testosterone, Estradiol and Albumin were measured on female samples using this test for SHBG, radioimmunoassays for total Testosterone and Estradiol and a Bromocresol Green colorimetric method for Albumin.

For the measurement of Total Testosterone, samples were extracted and chromatographied as previously described (13, 14). Testosterone was measured by RIA with Testosterone 1,2,6,7 ^{3}H as label and a rabbit anti-testosterone-3(*O*-carboxymethyl)-oxime - BSA (Bio-Merieux, Lyon, France). Intra-assay and inter-assay precision was 8.8 % and 10.8 %, respectively.

Estradiol was measured without extraction (15) using a commercially available kit (SORIN, Saluggia, Italy). Inter-assay precision was 7 %.

Using the values obtained, we calculated the Free Androgen Index (FAI) as follow (16) :

$$FAI = \frac{\text{Total Testo (nmol/l)}}{\text{SHBG (nmol/l)}} \times 100$$

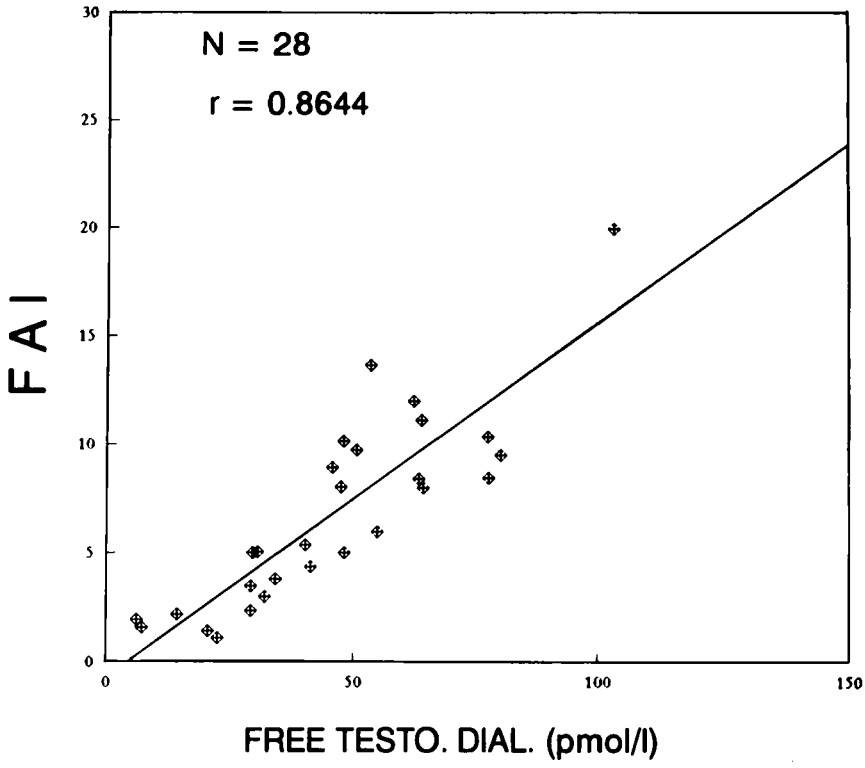

FIGURE 4. Correlation between Free Androgen Index and Free Testosterone measured by equilibrium dialysis.

According to the law of mass action (3,4), Free Testosterone was calculated using the values of SHBG, Total Testosterone, Estradiol and Albumin. For the calculation, we assumed one binding site on SHBG for either Testosterone or Estradiol. We also take count of the binding of each steroid to albumin. The following values for the association constants were used :

SHBG for Testosterone : $0.760.10^{-9}$ M^{-1}

SHBG for Estradiol : $0.314.10^{-9}$ M^{-1}

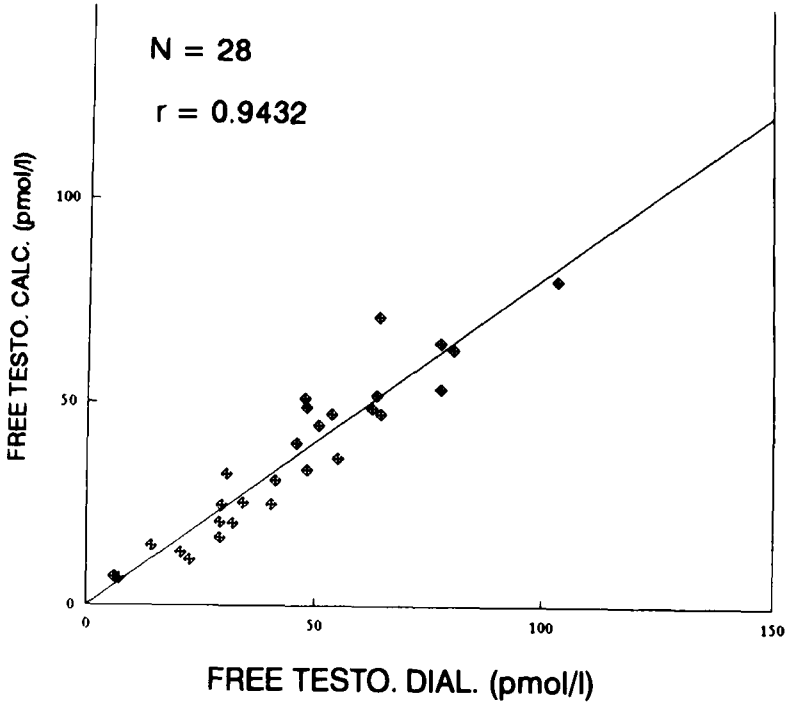

FIGURE 5. Correlation between Calculated Free Testosterone and Free Testosterone measured by equilibrium dialysis.

Albumin for Testosterone : $5.00.10^{-5}$ M^{-1}

Albumin for Estradiol : $4.21.10^{-5}$ M^{-1}

On the same samples, Free Testosterone was measured by equilibrium dialysis using ^{3}H Testosterone as previously described (17). FAI and Free Testosterone obtained by both methods were compared. The correlations are represented on Figures 4 and 5.

As it can be seen from the regression lines, there is a good agreement between the Free Androgen Index or the calculated Free Testosterone and

the Free Testosterone determined by equilibrium dialysis. It can also be noticed that the correlation is better when comparing calculated Free Testosterone with Free Testosterone by dialysis than FAI. This is probably due to the fact that the calculated FAI does not reflect the binding of Testosterone to Albumin and the competitive binding of Estradiol to SHBG.

DISCUSSION

This paper describes an immunoradiometric assay for the determination of Sex Hormone Binding Globulin (SHBG) in human serum. The method uses tubes coated with two monoclonal antibodies and a third monoclonal labeled with ^{125}I.

The detection limit is about 2.5 femtomol/tube with an incubation of 90 minutes at room temperature on an orbital shaker. Because the high sensitivity of this test, serum samples must be diluted 1:51 before analysis.

No "hook effect", which is sometimes a severe drawback of immunometric assays, has been found up to 1,000 nmol/l, which is far higher than the highest values encountered in human serum, for example in third trimester pregnancy serum.

This test has been used to calculate the Free Androgen Index and the Free Testosterone.

Using the SHBG values obtained by the present method, we observed good correlations between the calculated Free Androgen Index or the calculated Free Testosterone and the Free Testosterone measured by equilibrium dialysis although the correlation is better with calculated Free Testosterone than with FAI.

This measurement of SHBG together with a measurement of Total Testosterone and Estradiol can thus provide a good tool to assess a possible

hirsutism in women. Free Androgen Index or Free Testosterone calculations are easier to perform than the cumbersome and somewhat tedious measurement of Free Testosterone by equilibrium dialysis. Moreover, the calculated parameters obtained by this method using SHBG and Total Testosterone values should be better than a direct measurement of Free Testosterone by analog technique which has been severely criticized recently (18).

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