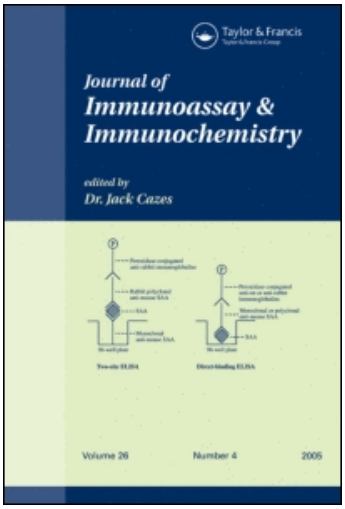

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Direct Radioimmunoassay for the Measurement of Serum Testosterone using ^{3}H as Label

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Abstract: Direct radioimmunoassay (RIA), based on the principle of competitive inhibition for the measurement of serum testosterone, using ^{3}H as label, is described. Testosterone 3-O-carboxymethyloxime-bovine serum albumin (testosterone 3-O-CMO-BSA) was used as an immunogen and testosterone, labeled at positions 1, 2, 6, and 7 with ^{3}H was used as tracer. To 12 × 75 mm glass tubes 100 μL of standard (250 to 10,000 pg/mL) and unknown samples were added in duplicate, followed by 100 μL of antibody and 600 μL of tracer (10,000 counts per minute [cpm]) in all the tubes and incubated overnight at 4°C. The bound and free fraction of labeled were separated by adding 200 μL of charcoal followed by centrifugation. The bound radioactivity was measured in the supernatant by using a scintillation fluid containing 2,5-diphenyloxazole (PPO, primary scintillator) and p-bis[2-(5-phenyloxazolyl)]-benzene (POPOP, secondary scintillator). In this new strategy, high ionic strength and low pH of the buffer are utilized to release bound steroid from proteins.

The sensitivity of the assay is 270 pg/mL. The analytical recovery ranged from 100.24% and 108.94%. The inter-assay and intra-assay coefficients of variation ranged from 3.38% to 9.56% and 5.69% to 9.84%, respectively. The serum testosterone values obtained by this method were correlated with those obtained by solid phase radioimmunoassay: $r = 0.91$ ($n = 34$).

Keywords: Testosterone, Direct radioimmunoassay, ^{3}H labeled immunoassay

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INTRODUCTION

The competition between plasma proteins and specific antibodies for an analyte is a well-documented phenomenon in the area of steroid immunoassay.[1,2] Steroids are reversibly bound to proteins in serum, and steroid immunoassays are usually classified according to the method by which this binding is overcome. Assays where the analyte is separated from other serum constituents prior to immunoassay, usually by solvent extraction, can produce analytically correct results which agree with results of methods such as mass spectrometry.[3] Direct (non-extraction) assays use chemical blocking/displacing agents with high affinity for serum protein binding sites to reduce, or ideally prevent, serum protein binding of the analyte.

The ^{3}H and ^{125}I radio-nuclei are generally being used in steroid RIAs. The ^{3}H tracers used in steroid RIA are non-analogue (chemically identical to the analyte), whereas ^{125}I tracers are of general structural analogue "steroid bridge label." Analogue steroid tracers are, thus, susceptible to bridge effects. All ^{3}H and ^{125}I labeled steroid analogue bind to serum proteins. The serum protein binding of ^{3}H labeled steroid were substantially reduced in all samples by addition of blocking agents, but the binding of ^{125}I labeled steroid analogue tracers in same samples were less affected. This suggests that serum protein binding of steroid analogue tracers may be a source of interference in some direct steroid immunoassays.[4]

Major steroids (e.g., cortisol/progesterone) bound in serum to corticosteroid binding globulin (CBG) may be displaced by protein binding agents such as 8-anilino-1-naphthalene sulphonic acid (8-ANS), and salicylate, by proteolytic enzyme, by low pH, or by heat treatment.[5] In addition, danazol, dexamethazone, and cortisol, which have affinities for CBG, have been used in direct assay for progesterone.[5,6] A disadvantage of these displacing agents is that, in their blocking concentration, some of them reduce the specific binding of the antigen with antibody.[2] In some cases, these displacing agents also cross-react with the antibody. In several cases, a mixture of different, non-related steroids is also used as displacing agent in direct assays of steroids, such as testosterone and estradiol. These exogenous steroids are potential sources of cross-reaction.[4,7] The complex mixtures of these displacing agents in direct immunoassay interfere with the performance of the assay and the sensitivity is affected.[6]

To overcome the problem of cross-reaction of blocking reagents with the specific antiserum and proper displacement of analyte and tracer from serum protein, a new strategy has been formulated. In this new strategy, high ionic strength and low pH of the buffer are utilized as to release bound steroid from proteins, which does not have any bearing on the specificity of the antiserum. The potential application of high ionic strength and low pH buffer is demonstrated through the development of a direct radioimmunoassay for the measurement of serum testosterone using ^{3}H as a label in human serum.

EXPERIMENTAL

Testosterone, testosterone-3-O-caboxymethyloxime (testosterone-3-O-CMO), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethyl-aminopropyl)-cabodiimide-HCl (EDAC), N-hydroxysuccinimide (NHS), Freund's complete adjuvant (FCA), thimerosal, activated charcoal, and dextran T-70 were all purchased from Sigma Chemical Company, St. Louis, MO., U.S.A. Testosterone with ^{3}H at position 1, 2, 6, and 7 was purchased from NEN life science product, Boston, MA., U.S.A. All other chemicals and buffer salts were of analytical grade.

Buffer

The buffers used were

a) 100 mM acetate buffer, pH 4.0 (CH_3COONa 8.2 gm/L and 7.4 mL of glacial acetic acid) containing 46 gm/L sucrose, 8.6 gm/L ammonium sulfate, 1 gm/L BSA, and 0.1 gm/L thimerosal.

b) 100 mM phosphate buffer, pH 7.2, ($NaH_2PO_4 \cdot 2H_2O$ 3.86 gm/L and $Na_2HPO_4 \cdot 2H_2O$ 10.2 gm/L) containing 46 gm/L sucrose, 8.6 gm/L ammonium sulfate, 1 gm/L BSA and 0.1 gm/L thimerosal.

Antibody Generation

Testosterone-3-O-CMO was covalently linked to BSA by an activated ester method and the New Zealand white rabbits were immunized with the conjugate according to the procedure described elsewhere.[8]

Titer

The antiserum was serially diluted with 100 mmol/L acetate buffer to give 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, and 1:16,000 dilutions. A volume of 100 μL of diluted antiserum was added in 12 mm X 75 mm glass tube induplicate, followed by the addition of radioactive [^{3}H]-testosterone (600 μL containing, 10,000 cpm) mixed and kept for overnight at 4°C. Separation of free and bound testosterone was achieved by adding 200 μL mL^{-1} of dextran coated charcoal suspension (0,625 gm activated charcoal and 0.0625 gm dextran T-70 in 100 mL acetate buffer). The reaction mixture was kept for 20 min and then centrifuged at 2,000 rpm for 10 min at 4°C. Supernatants containing the antibody bound [^{3}H]-testosterone were decanted into a scintillation vial containing 10 mL of scintillation fluid

(3gm of PPO and 0.1 gm of POPOP dissolved in one liter of toluene) and counted in a Wallac Liquid Scintillation Counter (Model 1409). The titer of the testosterone antiserum was defined as the dilution at which 50% of the tritiated testosterone was bound to the antibody.

Direct Radioimmunoassay using Acetate Buffer

To 12 mm × 75 mm glass tubes, 100 μL of standard (250 to 10,000 pgm/mL)/or unknown samples were added in duplicate, followed by 100 μL of antibody and 600 μL of tracer (10,000 cpm) in all of the tubes and incubated overnight at 4°C. Separation of free and bound testosterone was achieved by adding 200 μL of dextran coated charcoal suspension (0,625 gm activated charcoal and 0.0625 gm dextran T-70 in 100 mL acetate buffer). The reaction mixture was kept for 20 min and then centrifuged at 2,000 rpm for 10 min at 4°C. Supernatants containing the antibody bound [^{3}H]-testosterone were decanted into a scintillation vial containing 10 mL of scintillation fluid (3 gm of 2,5-diphenyloxazole (PPO, primary scintillator) and 0.1 gm of p-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP, secondary scintillator) dissolved in one liter of toluene, and counted in a Wallac Liquid Scintillation Counter (Model 1409).

Direct Radioimmunoassay using Phosphate Buffer

To 12 mm × 75 mm glass tubes, 100 μL of standard (250 to 10,000 pgm/mL)/or unknown samples were added in duplicate followed by 100 μL of antibody and 600 μL of tracer (10,000 cpm) in all of the tubes and incubated overnight at 4°C. Separation of free and bound testosterone was achieved by adding 200 μL of dextran coated charcoal suspension (0.625 gm activated charcoal and 0.0625 gm dextran T-70 in 100 mL phosphate buffer). The reaction mixture was kept for 20 min and then centrifuged at 2,000 rpm for 10 min at 4°C. Supernatants containing the antibody bound [^{3}H]-testosterone were decanted into a scintillation vial containing 10 mL of scintillation fluid (3 gm of 2,5-diphenyloxazole (PPO, primary scintillator) and 0.1 gm of p-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP, secondary scintillator) dissolved in one liter of toluene), and counted in a Wallac Liquid Scintillation Counter (Model 1409).

Solid Phase Radioimmunoassay

RIA of the samples was performed with a commercial RIA Kit (Immunotech, France). According to the manufacturer's assay protocol, 50 μL of standard or control or unextracted serum sample was dispensed to testosterone antibody

coated tubes in duplicate. Then, 500 μL of radioiodinated testosterone was added to all of the tubes and incubated at room temperature for 1 hour with moderate shaking. After incubation, the contents of the tubes were decanted and washed with water, thoroughly. The counts per minute were measured in a Wallac Automatic Gamma Counter (1470 WIZARD^{TM}).

Data Analysis

Quantification of the testosterone in the serum samples was performed by a in-house developed personal computer programme written in QBASIC language, using a logit-log linear regression method and the Mann-Whitney test was used to compare the values of the samples obtained by this direct RIA and solid phase RIA.

RESULTS

Sensitivity

The lowest detection limit of the assay, i.e., concentration equivalent to B_0 – 2SD was 270 pgm/mL of serum after thirty-fold determination of B_0 binding.

Specificity of Antibody

Testosterone antibody had less than 0.1% cross-reaction with naturally occurring C_{27}, C_{21}, C_{19}, and C_{18} steroids except 5α-dihydrotestosterone (10%). However, the cross reaction of nandrolone (a synthetic androgen) was 3.4%.

Analytical Recoveries

The ability of the assay to accurately quantify testosterone in serum samples was tested. Low, medium, and high concentration (1.0–10 ng/mL) of testosterone were added exogenously to three fractions of pooled serum. After addition, the concentration of testosterone was determined and the recovery was calculated in each serum fraction. The recovery ranged between 102.97% and 108.94% (Table 1), and 0% to 10% (Table 2) when measured by using acetate buffer and phosphate buffer, respectively.

Intra-Assay and Inter-Assay Variations

Table 3 indicates the precision profile of the acetate buffer based assay. The analysis of 4 quality control sera for intra-assay (n = 8, replicate of each

Table 1. Recovery of testosterone from exogenously spiked pooled serum using acetate buffer

	Testosterone added (ng/mL)	Expected (ng/mL)	Obtained (ng/mL)	Recovery (%)
Basal	0	—	3.09	—
Low	1	4.09	4.1	100.24
Medium	2.5	5.59	6.09	108.94
High	10	13.09	13.48	102.97

pool) gave CVs $< 9.56\%$ at all levels. The measured mean $\pm$ SD concentrations were as follows: serum A, 2.79 ± 0.25 (ng/mL), serum B, 3.44 ± 0.33 (ng/mL), serum C, 5.16 ± 0.40 (ng/mL), serum D, 11.81 ± 0.40 (ng/mL). Inter-assay CVs for these 4 sera in 6 separate assays (8 replicate of each pool) were $<9.84\%$ at all levels. The mean $\pm$ SD values of these samples were 2.56 ± 0.16, 3.23 ± 0.20, 4.92 ± 0.28, and 11.03 ± 0.63 ng/mL.

Comparison of Direct RIA with Solid Phase RIA for Testosterone Values

Testosterone values were measured in 34 serum samples by the acetate buffer based direct RIA and by a commercial solid phase RIA kit. Regression analysis of the samples yielded the following equation:

$$y(\text{Direct RIA}) = 1.45 \times (\text{solid phase RIA}) - 0.45; \quad r = 0.91$$

The values obtained by these two methods were again tested by the Mann-Whitney U Test procedure to compare the two methods. The two methods were identical, significantly ($Z = -0.769$, $p > 0.05$).

Table 2. Recovery of testosterone from exogenously spiked pooled serum using phosphate buffer

	Testosterone added (ng/mL)	Expected (ng/mL)	Obtained (ng/mL)	Recovery (%)
Basal	0	—	Not detectable	—
Low	1	Basal + 1	Not detectable	No recovery
Medium	2.5	Basal + 2.5	Not detectable	No recovery
High	10	Basal + 10	1 ng	10%

Table 3. Intra-assay and inter-assay CVs for measurement of serum testosteron

Mean ± SD ng/mL	CV%
Within assay (n = 8 each)	
2.79 ± 0.25	8.96
3.44 ± 0.33	9.56
5.16 ± 0.40	7.75
11.81 ± 0.40	3.38
Between assay (n = 6 each)	
2.56 ± 0.16	9.84
3.23 ± 0.16	6.19
4.92 ± 0.28	5.69
11.03 ± 0.63	5.71

DISCUSSION

The developed RIA for estimation of testosterone in human serum is direct and simple. Only 100 μL of serum is required and, within 2 days, the assay will be completed. In the present RIA procedure, the influence of ionic strength, along with pH of the buffer, have been investigated for the release of steroids bound to binding proteins. It was observed that a low pH (4.0) of buffer is responsible for the release of steroids from the binding proteins, as compared to high ionic strength along with neutral pH (7.2) of buffer. When recovery of exogenously spiked testosterone from serum was performed using phosphate buffer of pH 7.2, under estimation of added testosterone was observed. This may be due to binding of testosterone and its tracer to serum proteins and to assay design.

Serum testosterone assays play an important role in the clinical evaluation of a number of very common endocrine disorders. In males, testosterone assays are used primarily to confirm the diagnosis of hypogonadism, and also to evaluate boys with delayed or precocious puberty and to monitor the adequacy of testosterone therapy. Because the clinical manifestations of androgen deficiency are nonspecific, the presence of low serum testosterone levels in men with symptoms and findings consistent with androgen deficiency confirms the diagnosis of hypogonadism. In females, testosterone assays are used for evaluation of hyperandrogenism (e.g., idiopathic hirsutism, congenital adrenal hyperplasia, polycystic ovarian syndrome, and androgen-secreting ovarian or adrenal tumors) and, more recently, to diagnose androgen deficiency.

The routine clinical use of testosterone assays began approximately 30 yrs ago with the development of RIAs for testosterone that could be performed with relatively small quantities of blood following organic extraction and chromatographic separation.[9] Subsequently, there have been

remarkable advancements in immunoassays for testosterone, as well as for other hormones. Compared with the original RIAs, testosterone assays of today are more sensitive and specific, require smaller quantities of serum, do not involve extraction or chromatography, and are performed more rapidly and with less cost. In most large clinical chemistry and reference laboratories, testosterone assays are performed routinely with automated platforms, using non-radioactive methods.

The enhanced efficiency and reduced cost, improved sensitivity, ease of performance, and automation of modern testosterone assays have made them more available to clinicians and researchers, thereby facilitating both clinical care and research. Although the pace of these advances has been rapid, rigorous attention to accuracy of many hormone assays, including testosterone assays, has lagged behind and, in some instances, been overlooked.[10–12]

There are several possible regions for the lack of agreement between the results obtained with the immunoassays tested and isotope-dilution gas chromatography-mass spectrometry (ID/GC-MS) for human samples. The first is the so-called matrix effect: immunoassays were performed, as recommended by the manufacturers, with no extraction steps. Thus, certain compounds present in serum, such as lipids and proteins, specifically binding globulins [e.g., sex-hormone-binding globulin (SHBG)], may interfere with the immunoassay. This effect has been observed in isotopic immunoassay, although the authors of another study obtained conflicting results.[13–15] In a more recent study, Boots et al.[17] also reported such an effect. A similar effect has been observed with non-isotopic assays.[18] The interference involved in the matrix effect may contribute to the divergence of the results obtained by ID/GC-MS and immunoassays.

Another possible reason for the lack of agreement between the results obtained with specific polyclonal or monoclonal antibodies is that the antibodies did not seem sufficient to increase the specificity of the immunoassay. Indeed, direct immunoassays display cross-reactivity with structurally related steroids[19] or therapeutic agents that also interact with SHBG in the serum.[20] Interactions have also been observed between steroid tracers, the corresponding antibodies, and SHBG. Thus, the binding of other non-tritiated tracers to serum proteins may be a source of interference in some direct steroid immunoassays, depending on their affinity for SHBG and the antibody used.[4,14] Together, these factors lead to overestimation of steroid concentration in solid phase assays.

Other possible regions for the lack of agreement between the results obtained with the immunoassays tested and ID/GC-MS for human samples include the limit of detection and functional sensitivity, which are particularly important for the determination of low (<1.7 nmol/L) and very low (0.17 nmol/L) testosterone concentrations.[21,22]

The analytical variables of the present direct RIA, especially accuracy, which is regarded as a corner stone of the assay, are in agreement with the standardization of a method, which may be because of the use of high ionic strength and low pH of the assay buffer to release steroids bound to the binding proteins.

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