A sensitive enzyme-linked immunosorbent assay (ELISA) for testosterone: use of a novel heterologous hapten conjugated to penicillinase

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A microplate enzyme-linked immunosorbent assay (ELISA) has been developed for the measurement of testosterone in plasma. The assay uses a heterologous system consisting of a novel hapten 4-(17β-hydroxy-3-oxoestra-4,9-dien-11β-yl)butanoic acid ***(1)*** *conjugated to penicillinase (β-lactamase). The key reaction in the synthesis of the hapten was the cuprate-mediated 1,4-conjugate addition on 3,3,17,17-*bis*-ethylenedioxy-5α,10α-oxido-estr-9(11)-ene by the Grignard reagent derived from trimethyl 4-bromoorthobutyrate; this regiospecifically introduces the 11β-butanoate function. The hapten-penicillinase conjugate was used in the assay in conjunction with the immunoglobulin G (IgG) fraction derived from a previously characterized, highly specific, antitestosterone serum raised against a testosterone-19-O-carboxymethyl ether-bovine serum albumin (T-19-O-CME-BSA) conjugate. This unique system represents one incorporating three elements of structural heterology: bridge, site, and ring heterology between the antigen hapten and enzyme-linked hapten. The limit of detection was 10 pg of testosterone with a sensitivity range between 15 and 1,000 pg. A low level of cross-reactivity with 5α-dihydrotestosterone (6.17%) and 11β-hydroxytestosterone (1.03%) was noted. No interference was noted with other common androgens, estradiol, or progesterone. The sensitivity and selectivity observed in the assay may be attributable to the selection of penicillinase as the enzyme marker and the elements of conformational heterology between the antigen-linked and enzyme-conjugated steroid haptens.* (*Steroids* **57**:154–161, 1992)

Keywords: steroids; enzyme-linked immunosorbent assay; testosterone; penicillinase; 4-(17β-hydroxy-3-oxoestra-4,9-dien-11β-yl)butanoic acid

Introduction

Enzyme-linked immunosorbent assays (ELISAs) for the measurement of plasma testosterone have been developed by several investigators using a variety of combinations of haptens and enzymes. However, cross-reactivity with 5α-dihydrotestosterone (5α-DHT), a circulating androgen, has remained a persistent problem in the precision of testosterone enzyme immunoassays.

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Nambara and Hosoda have used C-4–linked testosterone hemiglutarate haptens and β-galactosidase combinations[1] but have observed an unacceptably high (62%) cross-reactivity with 5α-DHT. Tateishi et al.,[2] using testosterone-3-(*O*-carboxymethyl)oxime (T-3-*O*-CMO) hapten in a homologous assay have similarly observed a high cross-reactivity (63%) upon conjugation of the hapten to glucoamylase. Turkes et al.,[3] using an 11α-substituted hapten hooked up to the enzyme horseradish peroxidase obtained a somewhat lower but still significant cross-reaction of 23% with 5α-DHT. Joshi et al. have developed a homologous ELISA for testosterone using T-3-*O*-CMO hapten conjugated to penicillinase.[4] This system shows a 12.5% 5α-DHT cross-reactivity.

One approach to building in a greater sensitivity and

selectivity in steroidal enzyme immunoassays is to use heterologous combinations of haptens linked to the enzyme and antigen protein. Chemical heterology between such haptens may be reflected in either site-heterology where the hook-up to the antigen-protein and the enzyme is at different positions in the steroid nucleus, or bridge heterology wherein the chemical nature or length of the linking side-chain is different between the two haptens. Using such a strategy, Rassaie et al.[5] have recently developed a sensitive and specific ELISA for testosterone that shows no significant cross-reaction with 5α-DHT. In that assay, the hapten conjugated to the enzyme penicillinase was testosterone-11β-carboxymethyl ether (T-11β-CME) and that used to obtain the bovine serum albumin (BSA)-linked antigen was T-3-*O*-CMO. This system has both site- and bridge-heterologous elements to improve specificity and sensitivity. Rassaie et al. reported a sensitivity of 2.5 pg in a microplate assay system and 25 pg/tube in a tube assay with <0.005% 5α-DHT cross-reaction.

In the current study, we have used a previously reported, very specific antitestosterone serum raised against testosterone-19-*O*-carboxymethyl ether-BSA (T-19-*O*-CME-BSA) conjugate (Figure 1). This antiserum has been used very successfully in a sensitive and highly specific radioimmunoassay (RIA) for the measurement of testosterone in both male and female plasma without chromatography.[6] A new nonradiometric ELISA procedure has now been developed using a novel hapten **1** conjugated to penicillinase as the tracer (Figure 2). The hapten **1** represents one in which an additional element of chemical heterology has been incorporated—the unique ring fusion geometry between rings A and B in a 19-norsteroid with a Δ^9 double bond. This new hapten when conjugated to an enzyme is conformationally distinct from the T-19-*O*-CME hapten conjugated to BSA, yet provides the requisite antibody recognition features of testosterone.

The use of penicillinase is advantageous because of its high turnover rate, availability in consistently pure form, ease of detection of minute concentrations by a simple color reaction, and its absence in physiological fluids.[7] A recent study compared various enzyme labels in microtiter-plate immunoassays of progesterone[8] and found penicillinase to have optimal properties when compared with β-galactosidase, alkaline phosphatase, or peroxidase.

Penicillinase has been successfully used as a marker enzyme in the ELISAs developed for other steroid hormones: for estradiol[9] and cortisol.[10] A microplate ELISA now has been developed using this heterologous combination of antigen and enzyme-conjugate.

Experimental

Solvents and reagents used in chemical synthesis and buffer preparations were obtained from Aldrich Chemical Co., Milwaukee, WI, USA. Tetrahydrofuran was routinely dried and purified by distillation over $LiAlH_4$ immediately before use; other reagents were used without prior purification. Testosterone and steroid standards for cross-reactivity studies were purchased from Sigma Chemical Co., St. Louis, MO, USA, or from Steraloids, Inc., Wilton, NH, USA, and were checked for purity by thin-layer chromatography. Penicillinase (*Bacillus cereus* β-lactamase, EC 3.5.2.6), soluble starch, phenoxymethylpenicillin, and Sephadex G-25 beads for gel filtration were purchased from Sigma. BSA (RIA grade) was obtained from ICN Biochemicals, Cleveland, OH, USA. Microwell ELISA plates (96-well, polystyrene) were obtained from Corning (#25801). Melting points were determined in a Thomas-Hoover (Philadelphia, PA, USA) capillary apparatus and are uncorrected. Standard product-isolation procedure consisted of multiple extractions of crude reaction mixture into an organic solvent, washing with specific reagents and then with saturated sodium chloride solution, followed by drying the organic extract over anhydrous sodium sulfate and evaporation in vacuo. This is indicated by the phrase "product isolation" followed by a list of components in parentheses. Thin-layer chromatographic (TLC) analysis was performed on silica gel plates (250 μm) with fluorescent indicator (Uniplate, Analtech, Newark, DE, USA). Plates were viewed under short-wave ultraviolet (UV) light or by spraying with 15% perchloric acid solution, followed by charring. Flash chromatography was performed on silica gel columns (Merck & Co., Rahway, NJ, USA; grade 60, 230–400 mesh, 60 Å). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian 390 NMR, 90-MHz spectrometer (Sunnyvale, CA, USA), and values are reported in ppm (δ) values downfield from a tetramethylsilane standard. Mass spectra were recorded on a Finnigan-MAT SSQ 700 electron spray spectrometer (San José, CA, USA). Elemental analyses were obtained from Midwest Microlab, Indianapolis, IN, USA. Absorbances were read from the microtiter plates using a Dynatech MR 600 microplate reader with 630 nm filter (Dynatech Industries, McLean, VA, USA).

Chemical synthesis of hapten (1)

3,3,17,17-Bis(ethylenedioxy)-5α,10α-oxidoestr-9-ene (2). This compound was prepared by adaptation of the hexachloroacetone–hydrogen peroxide epoxidation procedure of Ottow et al.[11] for related 5,10 steroidal epoxides. Hexachloroacetone (1.22 g, 0.005 mole, Aldrich) was added to 3 ml of dichloromethane and the mixture was cooled to 0 C in an ice bath. Hydrogen peroxide solution (30%) w/v, 0.6 ml, 0.0053 mole) was added and the mixture was stirred for 10 minutes at 0 C. A solution of 3,3,17,17,bis-ethylenedioxyestra-5(10), 9(11)-diene[12,13] (1.5 g, 0.0042 mole) was added through a dropping funnel over a period of 3 minutes to the rapidly stirring ice cold mixture, which was stirred at 0 C for 2 hours, then warmed to ambient temperature and stirred overnight. Product isolation (dichloromethane solvent, 10% sodium sulfite, saturated sodium bicarbonate washes) yielded 1.48 g (95%), TLC (ethyl acetate/hexanes, 8:2) R_f = 0.59. NMR ($CDCl_3$) δ 0.86(s, 3 H, 18-CH_3), 3.9–4.0(m, 8 H, -O-CH_2-CH_2-O), 6.10(m, 1 H, 11-H). A small amount of a peak at 5.92 corresponding to the 5β,10β-epoxide was observed; a ratio of 7:3 of the 5α,10α:5β,10β epoxides was indicated in the product. A portion of the foam was exhaustively dried to yield a solid material, mp 106–109 C; literature mp 108–110 C.[13]

3,3,17,17-Bis(ethylenedioxy)-5α-hydroxy-11β-[4-(1-methoxycarbonyl)butyl]-estr-9-ene (3). In a 250-ml round-bottomed flask were added activated magnesium turnings (600 mg, 0.0246 mole), 25 ml of dry tetrahydrofuran, and a small crystal of iodine. A solution of trimethyl 4-bromoorthobutyrate (4.06 g, 0.0195 mole) and 1,2-dibromoethane entrainer (0.4 ml) were dissolved in 15 ml of dry tetrahydrofuran. Approximately 1.5 ml of this solution was added to the stirring magnesium metal at room temperature under a nitrogen atmosphere, the reaction was initiated, and the addition was continued dropwise, the rate of the addition being

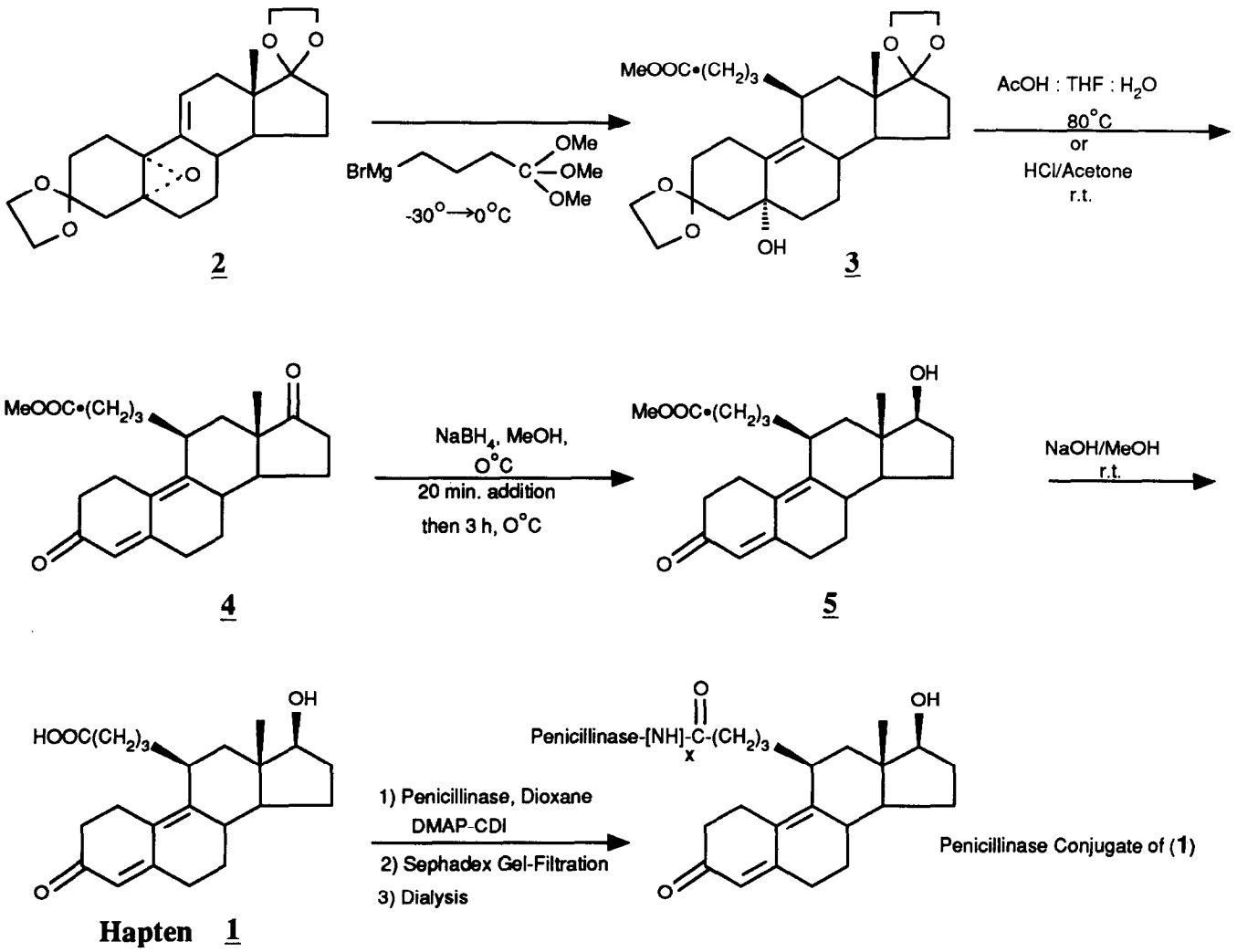

Figure 1 Synthesis of the hapten and its penicillinase conjugate.

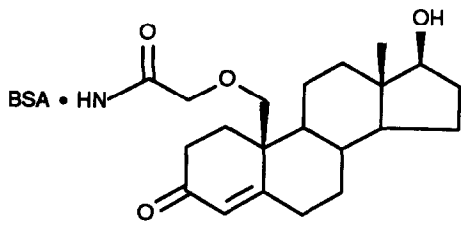

Figure 2 Antigen used for production of antibody. Heterology with hapten (**1**) is evident.

so adjusted as to keep the temperature below 40 C to minimize self-condensation of the Grignard reagent. The mixture was then stirred for 2 hours at ambient temperature providing a pale brown solution.

In a second, two-necked, 250-ml flask was added 25 ml of dry tetrahydrofuran in which the steroidal epoxide (**2**, 1.48 g, 0.004 mole) was dissolved. The solution was stirred and cooled to −30 C using an isopropanol–dry ice bath. Solid cuprous chloride (40 mg, 0.0004 mole) was added and the mixture was stirred for 20 minutes at −30 C. The Grignard reagent solution in tetrahydrofuran previously formed in the first flask was transferred via a double-tipped transfer needle to a dropping funnel attached to the flask containing the epoxide–cuprous chloride mixture. An additional 15 ml of tetrahydrofuran was added to the first flask and this was also transferred to the addition funnel under a stream of nitrogen. The Grignard reagent was added dropwise to the epoxide–CuCl mixture at −30 C to −10 C slowly over 1 hour and the reaction mixture was maintained at that temperature range for an additional 2.5 hours, when TLC (ethyl acetate/hexanes, 8 : 2) indicated absence of starting material **2**. The mixture was poured into 300 ml of a stirring mixture of 10% ammonium chloride solution and 100 ml of diethyl ether. The mixture was stirred at room temperature for 30 minutes to decompose the copper complex and then poured into a separatory funnel. Product isolation (ether solvent) yielded a viscous yellow oil. The oil was chromatographed by flash chromatography on a 400 × 30 mm column of silica gel; eluting with ether/hexanes (9 : 1). The by-product, dimethyl octan-1,8-dicarboxylate formed by self-coupling of the Grignard reagent, eluted first as a high R_f material. Subsequent elution provided fractions containing **3**, which were pooled and evaporated in vacuo to yield a colorless oil, 764 mg (40.6%). The oil resisted crystallization but was found to be homogeneous in TLC analysis (ether/hexanes, 9 : 1), R_f = 0.58; (Ether), R_f = 0.81. ^{1}H NMR ($CDCl_3$) δ 0.88(s, 3 H, 18-CH_3), 3.70(s, 3 H,

$COOCH_3$), 3.88–4.05(m, 8 H, -O-CH_2-CH_2-O). Calculated for $C_{26}H_{39}O_7$: C, 67.36; H, 8.47. Found: C, 67.14; H, 8.36.

11β-[4-(1-Methoxycarbonyl)butyl]-estra-4,9-diene-3,17-dione (4). The steroidal diketal ester (3, 682 mg, 0.00143 mole) was dissolved in anhydrous methanol (10 ml), concentrated HCl (0.2 ml) was added, and the mixture was stirred under a nitrogen atmosphere for 30 minutes at room temperature. Methanol was removed in vacuo under nitrogen; the residue was extracted into 100 ml of water. Product isolation (ethyl acetate solvent) yielded an oil: 481 mg (91%) of the diketone 4. TLC (diethyl ether, R_f = 0.57), ^{1}H-NMR ($CDCl_3$) δ 1.05(s, 3 H, 18-CH_3), 3.68(s, 3 H, $COOCH_3$), 5.73(s, 1 H, 4-H). Calculated for $C_{23}H_{30}O_4 \cdot 0.5\ H_2O$: C, 72.79; H, 8.24. Found: C, 72.67; H, 8.55.

11β-[4-(1-Methoxycarbonyl)butyl]-17β-hydroxyestra-4,9-dien-3-one (5). The steroidal dione (4, 0.481 g, 0.0013 mole) was dissolved in anhydrous methanol (20 ml) and the solution was cooled to 0 C in an ice bath. Sodium borohydride (80 mg, 0.02 mole) was added over 20 minutes and the mixture was stirred at 0 C. TLC monitoring (Et_2O) showed absence of starting material after a 3-hour reaction time at 0 C. The reaction was quenched by the dropwise addition of glacial acetic acid, and the methanol was removed from the mixture in vacuo under a nitrogen stream. The residue was extracted into 200 ml water. Product isolation (ethyl acetate solvent) yielded a colorless gum that showed low R_f non-UV active impurities on TLC (diethyl ether). The gum was purified by flash chromatography on silica gel (150 × 20 mm column) eluting with diethyl ether to provide 5 as a colorless gum, homogeneous on TLC, (diethyl ether) R_f = 0.43, 412 mg (85%). ^{1}H-NMR ($CDCl_3$) δ 0.92(s, 3 H, 18-CH_3), 3.67(s, 3 H, $COOCH_3$), 5.54(broad s, 1 H, OH, D_2O, exchangeable), 5.72(s, 1 H, 4-H). Calculated for $C_{23}H_{32}O_4$: C, 74.16; H, 8.66. Found: C, 73.98; H, 8.54.

4-[3-Oxo-17β-hydroxyestra-4,9-dien-11β-yl]butanoic acid (1). The steroidal methyl ester (5, 476 mg, 0.00128 mole) was dissolved in methanol (20 ml). A solution of sodium hydroxide granules (80 mg, 0.002 mole) in 2 ml of deionized water was added. The mixture was stirred under a nitrogen atmosphere for 3.5 hours, when TLC monitoring (diethyl ether) indicated disappearance of starting material. The reaction mixture was evaporated in vacuo under a stream of nitrogen. The residue was extracted into 50 ml of deionized water and acidified with 2 M sulfuric acid to precipitate a pale yellow solid. Product isolation (ethyl acetate solvent) gave a solid residue, mp 119–123 C. Recrystallized from acetone-hexanes, mp 121–123 C, 338 mg yield (73.8%). ^{1}H NMR ($CDCl_3$ + CD_3COCD_3) δ 1.01(s, 3 H, 18-CH_3), 3.68(dt, 2 H, $C\underline{H}_2$-COOH), 5.70(s, 1 H, 4 H). MS (m/z) 358 (M^+), 271 (M-87), 253 (M-105). Calculated for $C_{22}H_{30}O_4$: C, 73.71; H, 8.44. Found: C, 73.74; H, 8.46.

Preparation and purification of hapten (1)–penicillinase conjugate

The penicillinase conjugate of hapten 1 was prepared by an activated ester procedure as reported by Pandey et al.[9] for an estradiol-6-(O-carboxymethyl)oxime (E_2-6-CMO) hapten conjugated to penicillinase. Two milligrams (5.6 μmole) of 1 was dissolved in 250 μl of dioxane (HPLC grade) to which were added 0.805 mg (7 μmole) of *N*-hydroxysuccinimide and 1.760 mg (9.2 μmole) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (DMAP-CDI) hydrochloride. The mixture was allowed to stand at 4 C in a cold room for 6 hours. Dioxane was removed in vacuo under a stream of nitrogen and to the residue was added penicillinase [Sigma, type I, lyophilized powder containing 14% protein by Lowry assay, balance of citrate, and phosphate buffer salts (5.714 mg), equivalent of 800 μg of enzyme dissolved in 0.5 ml of 10 mM sodium phosphate buffered saline (pH 7.0), molar ratio of steroid to enzyme was 23] and the mixture was incubated at 4 C for 1 hour. The material was kept at −20 C overnight.

The following day the mixture was diluted with 1.5 ml of 10 mM sodium–phosphate-buffered saline (PBS), and was dialyzed in cellophane tubing against distilled water for 18 hours at 4 C. The dialysate was then applied to a Sephadex G-25 column (10 mm × 200 mm) and the mixture was gel-filtered, eluted with PBS buffer at 0 C under medium pressure using a peristaltic pump (Buchler Instruments, Fort Lee, NJ, USA), and 5-ml fractions were collected on a Beckman model 133A refrigerated fraction collector (Beckman Instruments, Fullerton, CA, USA). Eluted fractions were monitored for UV absorbance at 254 nm using a Uvicord KLB Model 4701 detector (Stockholm, Sweden). The penicillinase-hapten fraction eluted first, followed by a small amount of nonconjugated free enzyme. Low molecular weight organic impurities were retained on the gel and did not elute. Fractions having penicillinase activity were detected using a spot test in which 100 μl each of a solution of phenoxymethylpenicillin (0.0216 g in 50 ml of 10 mM PBS) was added to tubes. Twenty-five microliters from each eluted fraction was added to the tubes, which were incubated at room temperature for 1 hour. To each tube was added 500 μl of 5 N HCl to quench the enzyme reaction, followed by 0.5 ml of the starch-iodine reagent. Decolorization of the deep blue color of the starch iodine complex was indicative of fractions possessing penicillinase activity. The fractions were pooled, dialyzed against distilled water at 4 C for 16 hours, and stored at −20 C in 5 × 20 ml aliquots. The frozen conjugate solutions lost no enzymatic activity over a 9-month storage period.

Evaluation of the enzymatic activity of the hapten–penicillinase conjugate

Protein analysis of the enzyme–hapten conjugate[14] indicated that the conjugate stock solution contained 62 μg/ml of protein. The hapten–penicillinase conjugate was compared with an equivalent concentration of native nonconjugated penicillinase for its ability to hydrolyze the substrate phenoxymethylpenicillic acid.

Time course of the conjugate penicillinase reaction was compared with that of native enzyme by studying the rate of substrate hydrolysis, by plotting absorbance versus time curves for the enzymatic reaction using a fixed concentration of substrate (1.24 mM solution), and of enzyme or enzyme–hapten conjugate (250 μl of a 1.25 μg/ml solution). Substrate hydrolysis was followed by a color reaction as above. Results showed that the conjugate enzyme followed kinetics qualitatively very similar to the native enzyme and that conjugation did not significantly affect the enzyme reaction velocity (data not shown).

To determine if there was a quantitative difference in ability to hydrolyze substrate between modified and native enzymes, various concentrations of enzymes were used to hydrolyze the same concentration (1.25 mM) of substrate. The color reaction above was used and absorbances at various enzyme concentrations were recorded, reflecting the amount of substrate used. Plots of absorbance versus concentrations of free and conjugated enzymes (data not shown) showed near-parallel curves with similar slopes. On a weight-equivalent basis, the conjugated enzyme retained 72% of the activity of the native enzyme.

Preparation of immunoglobulin fraction of antiserum

The high-titer, antitestosterone serum previously characterized[6] was used to isolate the gamma globulin fraction, essentially by

the procedure of Levy and Sober[15] as modified by Shrivastav et al.[10] In brief, the antiserum was subjected to ammonium sulfate (33% w/v) precipitation, dissolution in 0.01 M PBS buffer, dialysis overnight at 4 C against 0.01 M ammonium carbonate buffer (pH 9.0), and lyophilization. The titer of the isolated IgG fraction was determined by charcoal-dextran RIA[6] to be a 1 : 75,000 dilution for inhibition of binding of 50 pmol of [1,2,6,7-^{3}H]testosterone.

Coating of microtiter plates

Initial coating procedures used a solution of 1 : 5,000 dilution of antitestosterone IgG fraction and using 0.02% glutaraldehyde in 0.01 M PBS buffer as the fixative, as described by Parsons.[16] Although this procedure gave suitable sensitivity and good standard curves in the ELISA, plates coated by this procedure could not be stored over 3 days at 4 C without loss of sensitivity, presumably due to cross-linking of antibody molecules over time. An alternate procedure using 0.1 M bicarbonate buffer (4.24 g Na_2CO_3 and 5.04 g $NaHCO_3$ in 1 L deionized water, pH 9.6) was then adopted, which provided plates that could be stored without loss of sensitivity for over a month. Plates were precoated with 250 μl of 0.1% BSA–PBS buffer. The buffer was decanted and 250 μl of antitestosterone IgG solution (1 : 5,000 dilution was found to be the best working dilution by a checkerboard titration) was added to each well. Plates were incubated for 3 hours at room temperature and then overnight (15 hours) at 4 C. The wells were washed several times with distilled water using a Corning 26305 plate washer. Remaining binding sites were blocked by addition of 200 μl of 0.1% BSA in 0.01 M PBS to each well, then incubated for 2 hours at room temperature. The wells were then drained and washed with distilled water, and the plates were stored covered at 4 C until use.

Microplate enzyme immunoassay

To each well of the 96-well microtiter plate were added 100 μl of testosterone standard in concentrations ranging from 10 to 1,000 pg, 100 μl of hapten–penicillinase conjugate (1 : 100 working dilution of stock solution), and 100 μl of 0.2 M sodium phosphate buffer. The first two rows of eight wells each were not coated with antibody and were used to assess nonspecific binding. The plates were incubated at room temperature for 2.5 hours. The contents of the wells were drained and the plate was washed three times with deionized water, then drained again. To each well was added 50 μl of a freshly prepared solution of phenoxymethyl penicillin sodium salt (1.5 M in 0.2 M sodium phosphate buffer, pH 7.2) and the plates were incubated at room temperature for 1 hour. To each well was added 200 μl of starch-iodine reagent, followed by 50 μl of 6 N HCl. The optical densities of the solutions in the wells were read on the microplate reader using a 630-nm filter. In the cross-reactivity experiments, the above procedure was followed replacing testosterone standard with 100 μl of concentrations of the appropriate test steroid. In the experiments where testosterone was assayed in the presence of cross-reacting steroid 5α-DHT, 100 μl each of the testosterone standards and 100 μl (containing 1 ng) of 5α-DHT standard solution was used for the initial incubation together with 100 μl per well of penicillinase conjugate. The additional 100 μl of 0.2 M sodium phosphate buffer was omitted.

Buffers and reagents

0.2 M sodium phosphate buffer, pH 7.2. 0.1 M phosphate buffered saline (PBS buffer). BSA assay buffer: 0.1% BSA added to 0.1 M sodium phosphate buffered saline. Starch–iodine reagent: 0.55 ml of iodine solution (0.18 M I_2 in 3.2 M KI in distilled water) added to 30 ml of 2% hydrolyzed starch solution. Made up to 200 ml in distilled water. Solution must be made up fresh daily.

Results

Synthesis of the hapten

The synthesis of **1** commenced with the 5α,10α-oxido steroid **2** (Figure 2). This compound could be generated from 3,3,17,17-(bis)ethylenedioxyestra-5(10), 9(11)-diene with the greatest stereoselectivity using the combination of reagents hexachloroacetone and hydrogen peroxide at 0 C[11] giving a >7 : 3 ratio of 5α,10α : 5β,10β epoxides. Other epoxidation routes using 3-chloroperoxybenzoic acid[17] or diethyl chloroformate-hydrogen peroxide[18] proved less satisfactory, yielding unfavorable isomer ratios and additional by-products.

A 1,4-conjugate addition on **2** using the Grignard reagent derived from methyl 4-bromoorthobutyrate with CuBr-Me_2S mediation[13] proved unsatisfactory, yielding a large amount of the cross-coupled product of the bromo reactant, namely dimethyl suberate, incomplete reaction with low yield, and recovery of starting material **2**. However, an inverse-addition procedure at −30 C using CuCl catalysis produced a clean reaction; the approach is analogous to that reported by Bélanger et al.[19] The reaction provided **3**; the ortho-ester function did not survive the Grignard reaction, presumably being hydrolyzed during the workup procedure to the methyl ester. Diketal deprotection of **3** under mild aqueous acid conditions was accompanied by dehydration to generate the 3-oxo-4-ene system of **4**. This product was then subjected to a mild sodium borohydride reduction in methanol under which conditions the 17-keto group was readily reduced to the 17β-alcohol **5**.[6] Under the conditions used, the methyl ester group and 3-keto function remained unaffected. Finally, a saponification of **5** provided the required hapten **1**.

Characteristics of the penicillinase–hapten conjugate

The conjugate retained the ability to effectively hydrolyze the substrate, phenoxymethylpenicillin. The molar ratio of enzyme (mol wt 31,000) and the steroid hapten used in the conjugation was 1 : 23. The purified conjugate retained full enzymatic activity over a period of 9 months when stored at +20 C.

Testosterone ELISA standard curve

A typical standard curve in the assay is shown in Figure 3. The assay was sensitive over a concentration range of 15–1,000 pg of testosterone. The lowest detectable limit distinguishable from control by the color reaction was 10 pg per well. Curve fitting was obtained using a Graph program (Micromath Scientific Software, Salt Lake City, UT, USA) for plotting and data transformation.

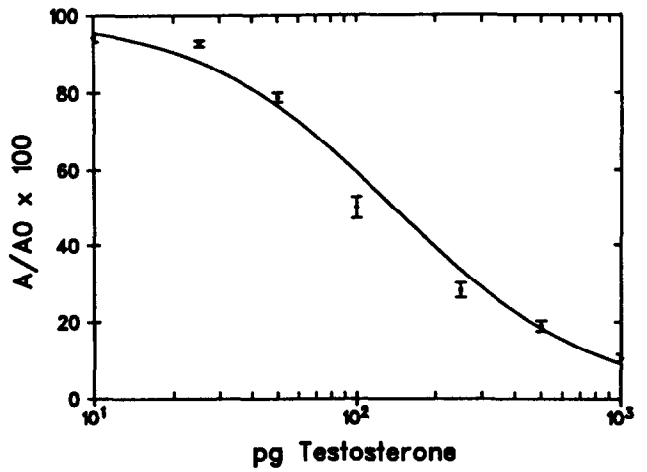

Figure 3 Typical standard curve for the enzyme immunoassay of testosterone.

Table 1 Cross-reactivity of androgens, estrogens, and progestin in the testosterone ELISA

Steroid	% Cross-reactivity[a]
Testosterone	100.00
5α-Dihydrotestosterone	6.17
Androstenedione	0.00
5α-Androstane-3β,17β-diol	0.00
11β-Hydroxytestosterone	1.03
Estradiol	0.00
Progesterone	0.00

[a] Defined as $100X/Y$ where X is amount in picograms (testosterone) and Y is amount in picograms (cross-reacting steroid) required to produce 50% inhibition of binding of enzyme-labeled testosterone hapten to antibody.

Specificity of the assay

The percent cross-reaction of related androgens and estrogenic steroids in the assay was determined by performing the microplate assay using 200 μl of the steroid solution at varying concentrations in place of the testosterone standard. Minor cross-reaction was observed with 5α-DHT (6.17%) and 11β-hydroxytestosterone (1%). The other steroids tested showed practically no cross-reactivity (Table 1).

Accuracy of the assay

Recoveries of added testosterone to charcoal-stripped human plasma. The recoveries of exogenously added concentrations of testosterone (100, 150, 300, and 700 pg) to charcoal stripped plasma in the assay are shown in Table 2. Recoveries ranged between 90 and 109%.

Recoveries of added testosterone in presence of cross-reacting steroid 5α-DHT. Recoveries of exogenously added concentrations of testosterone to charcoal-stripped plasma in the presence of 1 ng per well of 5α-DHT ranged between 96 and 118% (Table 3).

Precision of the assay

The typical intraassay coefficient of variation at various concentrations for testosterone measurement is shown in Table 2. Interassay coefficients of variation for six assays at specified testosterone concentrations were 25 pg, 7.2%; 100 pg, 6.8%; 250 pg, 9.4%; and 1,000 pg, 6.9%.

Discussion

A heterologous combination of haptens conjugated to enzyme (penicillinase) and antigen protein (BSA) was used to validate a new ELISA procedure for plasma testosterone. The assay was sensitive over a wide range of testosterone concentrations (15–1,000 pg), with 5α-DHT being the only cross-reacting androgen of significance. However, this value is considerably lower than the cross-reactivities reported in earlier enzyme-immunoassays of testosterone.[1–4] The assay therefore represents a sensitive and specific assay for testosterone in plasma adapted for use in microtiter plates.

The hapten **1** conjugated to penicillinase is structurally unique as a testosterone hapten in being a 19-norsteroid with a Δ^9 double bond and 11β-substitution. This novel hapten represents three elements of heterology when its structure is compared with the testosterone-19-*O*-carboxymethyl antigen conjugated to BSA, namely site, bridge, and ring heterology.

The influence of heterologous combinations of enzyme-linked hapten and antigen-linked haptens in steroid hormone ELISAs was first recognized by van Weemen and Schuurs.[20] Hosoda et al.[21] have found that the length and nature of bridging groups in haptens were important determinants of sensitivity in an ELISA for 11-deoxycortisol.

Sauer et al.[22] have made a similar deduction in the use of bridge-heterologous haptens in an ELISA for progesterone in milk, and have found the choice of enzyme marker to be an important factor as well.[8]

Because ELISAs are generally more rapid (when compared with an overnight incubation at 4 C in most charcoal-dextran RIAs), and are performed at room temperature or 37 C, they are generally non–equilibrium-binding assays. The choice of enzyme label therefore is an important determinant of sensitivity. It has been established that the lower the molecular weight of the enzyme–steroid conjugate, the more favorable are the binding kinetics.[22] In this context, penicillinase, a relatively low molecular weight enzyme (31,000) has an advantage over other enzymes such as β-galactosidase or alkaline phosphatase. Its other advantages include its ready availability in consistently pure form, ease of detection at extremely low concentrations, stability of the enzyme–steroid conjugate, high turnover rate, and absence in physiological fluids.[7] Thus, a combination of heterologous haptens and penicillinase as

Table 2 Recoveries of testosterone added to charcoal-stripped human plasma

Testosterone added (pg)	Testosterone measured (pg)[a]		Coefficient of variation (%)	Mean % recovery
	Mean	Range		
100	94	90–108	9.6	94.0
150	148	139–157	3.7	98.7
300	310	288–326	2.6	103.3
700	722	671–745	3.4	103.1

[a] Results of eight replicate experiments.

Table 3 Recoveries of testosterone added to charcoal-stripped plasma in presence of 1 ng 5α-DHT

Testosterone added (pg)	Testosterone measured (pg)[a]		Coefficient of variation (%)	Mean % recovery
	Mean	Range		
100	112	102–118	9.4	112.0
150	154	142–161	3.2	103.6
300	308	279–324	4.6	102.0
700	694	658–734	10.2	96.1

[a] Results of eight replicate experiments.

enzyme marker can lead to a sensitive and specific ELISA for plasma testosterone.

The present ELISA compares well in its sensitivity and specificity to a testosterone RIA developed in this laboratory[6] using the same antiserum raised against T-19-O-CME as the antibody source. This antiserum has been used for assay of testosterone in both male and female clinical samples. The use of this antiserum has now also provided a sensitive nonradiometric ELISA for testosterone using a novel hapten–enzyme tracer.

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