A SPECIFIC RADIOIMMUNOASSAY FOR ANDROSTENEDIONE WITH REDUCED BRIDGE-BINDING

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ABSTRACT

Antibody used in a steroid radioimmunoassay raised against a steroid hapten-carrier protein conjugate may recognize both the hapten and the chemical bridge to the protein. Use of the same bridge in the radioisotopic label may lead to higher affinity binding to the label than to the native steroid. Inhibition curves under these conditions are shallow and generally not acceptable for radioimmunoassay procedures. We have developed a radioimmunoassay for androstenedione that employs different bridges at the 11β position of the steroid for the protein conjugate and label. The resulting assay has greatly reduced bridge-binding, has an acceptable slope for the standard curve and is very specific as evidenced by low crossreactivies to other steroids.

INTRODUCTION

To develop a radioimmunoassay (RIA) for measuring steroids, antibodies for the assay are produced using a steroid hapten-carrier protein conjugate. The resulting antibodies often recognize and demonstrate high affinity binding to both the steroid and the chemical bridge through which the hapten was attached to the carrier protein. This phenomena is referred to as bridge-binding (1-8). Use of ^{125}I as the radionuclide to produce a labeled trace for the RIA requires that the iodinated functional group, phenol or imidazole, be attached to the steroid with a chemical bridge. However, if the label-bridge is homologous to the conjugate-bridge, the antibodies will have a higher affinity for the label than the native steroid. Consequently, the amount of steroid required to displace the radioiodinated steroid will be so great that the standard curve will shift to the right resulting in a substantial loss of sensitivity. In addition, the slope of the curve will be very shallow causing a loss of precision.

Recently we reported (1) on a procedure for reducing bridge-binding in a RIA for androstenedione (9). By using both a succinyl ester linkage ($-O_2CCH_2CH_2CO_2$-steroid) and an ether linkage ($-NHCOCH_2O$-steroid) attached to the 19 position of androstenedione, we were able to obtain a very sensitive and precise RIA. Unfortunately, the antisera, which was produced in sheep, was not very specific. The assay had a high crossreactivity towards closely related steroids, such as 5α-androstanedione, dehydroepiandrosterone and androsterone. Later, Rao et al reported (10) a RIA using a tritiated label and rabbit antibody against a bovine serum albumin-androstenedione conjugate (BSA-$NHCOCH_2O$-19-androstenedione) which was structurally identical to one that we had used (1). Although their rabbit antibody was more specific than the sheep antisera, it exhibited high nonspecificity to 5α-androstanedione and crossreactivity of about 2% or more to testosterone, dehydroepiandrosterone and androsterone.

We wish to report a RIA that employs sheep antibody against androstenedione linked to BSA with an ether bridge at the 11β position of the steroid (BSA-$NHCOCH_2O$-11β-androstenedione) and a label that has an ester bridge (^{125}I-phenol-CH_2CO_2-11β-androstenedione). The resulting assay is very sensitive and precise. In addition, the crossreactivities of the antibody against testosterone, androsterone and dehydroepiandrosterone are at least ten-fold less than those reported by Rao et al (10).

MATERIALS

$Na[^{125}I]$ (carrier free) was obtained from New England Nuclear. 11β-hydroxyandrostenedione, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, dehydroepiandrosterone, testosterone, 5α-dihydrotestosterone, pigskin gelatin, bovine serum albumin and merthiolate (thimerosal) were obtained from Sigma Chemical Co. Ethyl diazoacetate, p-toluenesulfonic acid and p-hydroxyphenylacetic acid were purchased from Aldrich Chemical Co. Chloramine-T and sodium metabisulfite were from J.T. Baker Co. Androstenedione, androsterone, 5α-androstenedione, progesterone and estradiol-17β were purchased from Steraloids Inc., Wilton, N.H. $RhCl_3.3H_2O$ was from Ventron Corporation and HPLC grade acetonitrile was from Burdick & Jackson Laboratories, Muskegon, MI. Other solvents were analytical grade. Steroids used as RIA standards and crossreactants were recrystallized.

SYNTHESES

The syntheses of the conjugate and radioiodinated label used in this study are outlined in figure 1. Melting points were determined

in capillary tubes in a Thomas-Hoover apparatus and were corrected. Spectral analyses were obtained with the following instruments: IR, Perkin-Elmer 281; NMR, Varian EM-360; and UV, Cary 219.

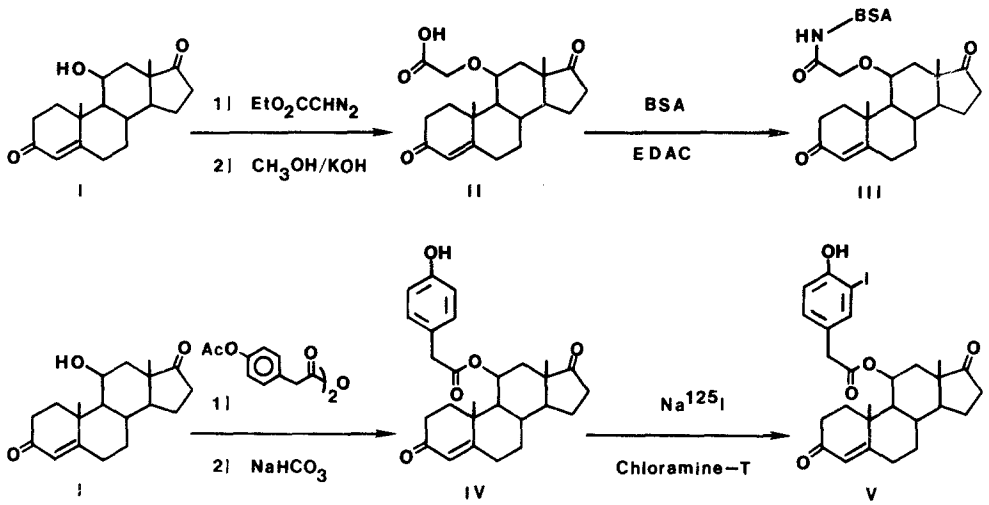

Figure 1. Synthetic Schemes

11β-(Carboxymethoxy)-4-androstene-3,17-dione (II). 11β-hydroxy-androstenedione (250 mg, 0.84 mmol) was dissolved in dry benzene (40 mL). The catalyst $Rh_2(O_2CCH_3)_4$ (3 mg), prepared from $RhCl_3.H_2O$ (11), was then added. Ethyl diazoacetate (1.09 gm, 9.51 mmol) was added dropwise to the steroid solution. Additional ethyl diazoacetate (2.18 gm, 19.02 mmol) was added 6 hr later, and the reaction stirred at room temperature overnight. The reaction mixture was poured into H_2O (100 mL) and extracted five times with $CHCl_3$ (50 mL). The combined organic layer was dried with Na_2SO_4 and evaporated *in vacuo* yielding a yellow oil. The oil was chromatographed on a 1.5 x 30 cm silica gel column using a 0 to 2% CH_3OH gradient in $CHCl_3$ for elution. A second chromatography step on a 1.5 x 30 cm silica gel column with a 0 to 20% ethyl acetate gradient yielded 11β-(ethylcarboxymethoxy)-4-androstene-3,17-dione as a clear oil (120 mg, 37.4%). IR ($CHCl_3$), ν_{max} 1740 and 1668 cm^{-1}: NMR ($CDCl_3$),δ 1.10 (s,3H,18-CH_3), 1.26 (t J=8.0 cps,3H, -OCH_2CH_3), 1.52 (s,3H,19-CH_3), 4.10 (m,5H,11β-OCH_2CO_2-,-OCH_2CH_3, 11α-H), 5.72 (s,1H,4-H). Anal. Calc. $C_{23}H_{32}O_5$: C,71.11;H,8.30. Found: C,71.15;H,8.26.

The ethylcarboxymethoxy derivative from above (135 mg, 0.346 mmol) was dissolved in CH_3OH (10 mL). KOH (6.4 mL of 0.1 M, 0.70 mmol) was added dropwise at 10°C. The reaction mixture was then poured into saturated NaCl solution (200 mL) and made slightly acidic with 1 N HCl. The resulting aqueous mixture was extracted six times with $CHCl_3$ (10 mL). The combined organic phase was dried as above and evaporated in vacuo yielding 11β-(carboxymethoxy)-androstene-3,17-dione (II) as a clear oil (120 mg, 96%). IR ($CHCl_3$), νmax 3400, 1739 and 1668 cm^{-1}: NMR ($CDCl_3$), δ 1.07 (s,3H,18-CH_3), 1.50 (s,3H, 19-CH_3), 4.05 (s,2H, -OCH_2CO_2H), 4.18 (m,1H,11α-H), 5.75 (s,1H,4-H), 6.00 (broad s,1H, -CO2H,exchanged with D_2O).

11β-[N-(Bovine serum albumin)-amidocarboxymethoxy]-4-androstene-3,17-dione (III). The carboxymethoxy derivative II (100 mg, 0.28 mmol) was dissolved in dimethyl formamide (10 mL) and H_2O (4 mL). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) (100 mg, 1.92 mmol was added, and the mixture stirred for 30 min. Bovine serum albumin (BSA) (200 mg) dissolved in phosphate buffer (0.01 M, pH 7.8, 20 mL) was added dropwise to the steroid solution which was stirred two days at ambient temperature in the dark. The solution was dialyzed against 0.05 M $NaHCO_3$ (2 x 2 L) and H_2O (4 x 2 L) and lyophilized. The molar ratio of the steroid to protein was found to be 15.0 by spectral analysis (1).

11β-(p-Hydroxyphenylacetoxy)-4-androstene-3,17-dione (IV). p-Toluenesulfonic acid (160 mg, 0.842 mmol) was dissolved in benzene (30 mL), and the mixture refluxed with a Dean-Stark apparatus for 3 hr to remove H_2O. 11β-Hydroxyandrostenedione (100 mg, 0.33 mmol) and p-acetoxyphenylacetic anhydride (1) (200 mg, 0.54 mmol) were added to the benzene solution and the resulting mixture was refluxed for 10 additional min, cooled to room temperature and stirred 48 hr. The reaction mixture was poured into H_2O (100 mL) and extracted six times with $CHCl_3$ (30 mL). The combined organic layer was dried as before and evaporated in vacuo yielding a clear oil. The oil was dissolved in methanol (5 mL), 4 drops of saturated $NaHCO_3$ were added, and the mixture stirred fo 1.5 hr. The pH of the solution was adjusted to 2.0 with 0.5 N HCl and extracted five times with $CHCl_3$ (15 mL). The chloroform layer was washed twice with H_2O (10 mL) and dried. Removal of the solvent left a yellow oil that was purified on a 1.5 x 30 cm silica gel column. Elution with benzene/ethyl acetate (4/1) yielded a clear oil. Recrystallization from acetone/hexane produced IV as a white solid (84 mg, 58%) mp 154-155°C. IR (CH_2Cl_2), νmax 3600, 1730, 1670 and 1525 cm^{-1}: NMR ($CDCl_3$), δ 0.78 (s,3H,18-CH_3), 1.14 (s,3H, 19-CH_3), 3.50 (s,2H,phenyl-CH_2CO_2-), 4.49 (m,1H,11α-H), 5.71 (s,1H, 4-H), 6.76 and 7.11 (dd J=10.5 cps,4H,phenyl-H), 7.80 (broad s,1H, phenyl-OH, exchanged with D_2O). Anal. Calc. $C_{27}H_{32}O_5$: C, 74.29; H, 7.39. Found: C, 74.41; H, 7.46.

Radioiodination of IV. The p-hydroxyphenyl derivative IV (2.0 ug, 4.58 nmol) in ethanol (2 uL) was placed in a 1 mL vial fitted with a multi-dose septum. Phosphate buffer (0.5 M, pH 7.4, 40 uL) and aqueous Na[^{125}I] (4.0 mCi, 11.2 uL) were added. The iodination was initiated by the addition of chloramine-T (20 ug, 106.5 nmol) in phosphate buffer (0.05 M, pH 7.4, 30 uL) (buffer A). After shaking at

ambient temperature for 1 min, the reaction was quenched by the addition of sodium metabisulfite (10 ug, 52.5 nmol) in buffer A (20 uL). Purification of the radioiodinated steroid was done by HPLC. The entire reaction mixture was injected onto an Altech 600-RPA C_{18} reverse phase column using a Rheodyne injector fitted with a 100 uL loop. A Spectro Physics SP8700 pump provided the following mobile phase at a flow rate of 1 mL/min: 0 to 5 min, an isocratic mixture of 20% acetonitrile in 0.05 M potassium formate buffer, pH 4.0; 5 to 80 min, a 20 to 60% gradient of acetonitrile in the formate buffer. The eluted material was collected in a Gilson FC-80 Fraction Collector (1 min fractions), and the radioactivity was quantitated with a gamma counter. Both a monoiodinated (eluting at 65 min) and a diiodinated derivative (77 min) of the steroid were isolated. The estimated specific activity (12) of the two labels were found to be 3.3 mCi/ug and 5.4 mCi/ug, respectively . The monoiodinated label, which was used in the RIA, had a maximal binding (active fraction) of 98% in the presence of exess antibody.

METHODS

Antibody Production. Antibody against the steroid-BSA conjugate III was raised in five Suffolk ewes. Two milligrams of the conjugate in Freund's complete adjuvant (1.0 mL) was injected intradermally at multiple sites on the flanks of each animal. Booster injections of 1.0 mg in incomplete Fruend's adjuvant (1.0 mL) were given at weeks 4 and 8. Bleedings were taken at weeks 10 and 11 and the highest titers (dilution of serum needed to bind 50% of the label) were found at week 11. Serum from sheep #95, which had the best specificity, was used for the RIA.

Radioimmunoassay. The radioimmunoassay was run as described previously (1) using a 1/50,000 dilution of antisera and 40,000 cpm/tube of label. Crossreactivity studies were done using the method of Thorneycroft et al (13).

RESULTS AND DISCUSSION

It was obvious from our previous paper (1) that bridge-binding could be significantly reduced in a RIA for androstenedione by employing heterologous bridges when synthesizing the immunogenic steroid-carrier protein conjugate and the ^{125}I-steroid label. It was also apparent that use of the 19 position of the steroid for functionalization introduced problems of specificity. Consequently, we chose to use the same type of linkages but at a different position, namely, the 11β position of androstenedione. When antibody against an ether-linked BSA conjugate (BSA-$NHCOCH_2O$-11β-androstenedione) was used in conjunction with an ester-linked label (^{125}I-phenol-CH_2CO_2-11β-androstenedione), we obtained a standard curve with a 50% inhibition point

of 104 pg/tube and a slope of -1.73. The 50% point was somewhat higher than that reported previously (1) (104 pg vs. 15 or 76 pg). However, the actual limit of detection (14) is certainly adequate for routine use (200 femtograms (fg)/assay tube). The slope of -1.73 was less than the ideal -2.303 for a logit-log curve but provides a workable assay.

The most obvious improvement was in assay specificity as can be seen in table 1 which compares the crossreactivity of our current assay using antibody against the 11β-conjugate to the crossreactivities we reported (1) and those Rao et al reported (10) using antibodies to the 19-conjugate.

Table 1

	%Crossreactivity		
Steroid	Anti-11	Anti-19 (1)	Rao et al (10)
Androstenedione	100.0	100.0	100.0
5α-Androstanedione	5.2	61.7	30.38
Testosterone	0.06	0.05	2.50
5α-Dihydrotestosterone	0.17	0.04	1.55
Dehydroepiandrosterone	0.21	10.0	2.54
Androsterone	0.14	48.1	1.93
Progesterone	0.26	0.03	0.55
Estradiol-17β	0.007	0.08	0.08

The crossreactivity of the 11β-system to 5α-androstanedione is 5.2% vs. 61.7% and 30.38% for the two 19-systems. Antibodies to androgens, such as androstenedione and testosterone, usually cannot differentiate very well between the 4-ene and 5α- forms of the steroids (15-18). The best evidence for improved specificity lies in the comparison of the crossreactivities of dehydroepiandrosterone and androsterone. We obtained 0.21% and 0.14%, respectively, compared to 10.0% and 48.1% for the anti-19-conjugate (1). Although Rao et al (10) reported antisera (rabbit) to the 19-conjugate with higher specificity than we described (sheep) using the same immunogen (1), the rabbit antibodies are at least 10-fold less specific than the anti-11β-conjugate antibodies. In addition, the anti-11β-antiserum also has lower crossreactivity to testosterone and 5α-dihydrotestosterone than that reported for the rabbit anti-19-conjugate (10).

The crossreactivity of anti-androstenedione antisera to testosterone and dehydroepiandrosterone is critical since they are present in male human serum samples at concentrations greater than or equal to the amount of androstenedione (19) and would interfere in a RIA.

In conclusion, by using the heterologous bridges we described previously (1), a sensitive and precise steroid radioimmunoassay can be developed. This is evidenced by the assay limit of detection of 200 fg/tube and slope of the inhibition curve of -1.73. In addition, one can produce very specific antibodies by using the 11β position of androstenedione for attachment of the carrier-protein and labeled functional groups.

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