A CHEMICAL APPROACH TO SOLVING BRIDGING PHENOMENA IN STEROID RADIOIMMUNOASSAYS

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ABSTRACT

Steroid radioimmunoassays (RIA) employ antibodies raised against a carrier protein-steroid conjugate. Individual antibodies may recognize the steroid, the protein or the chemical bridge used to join them together. Use of the same bridge in the tracer results in higher affinity binding of the tracer than the native ligand which in turn results in a loss of sensitivity and precision. We have greatly reduced bridge-binding in a RIA for androstenedione. Conjugates and radioiodinated labels were prepared with either an ester or ether chemical bridge. By using an antibody and the corresponding label with the heterologous bridge very sensitive assays were obtained.

INTRODUCTION

The major advantages of using γ-emitting radionuclides such as ^{125}I as opposed to tritium or other β-emitting radioisotopes to label tracers for steroid RIAs are: 1) the higher specific activity of the γ-emitting radiolabeled tracers (1), and 2) the ease and cost effective manner in which samples can be counted. The net effect of both of these factors is that more sensitive, less costly steroid assays are possible with γ-emitting tracers than with tritium based assays. The most serious

disadvantage to using radioiodinated labels arises from the affinity that the antibody may show not only for the hapten but for the bridge through which it was attached to the carrier protein. The recognition of the chemical bridge by the antibody has been refered to as bridge-binding (1-6). The problem arises when a radioiodinated histamine, tyrosine methyl ester or tyramine moiety is attached to the steroid through the same chemical bridge that was used to attach the hapten to the carrier protein in the immunogen. Because of the homology of the bridges, the antibody recognizes both the steroid and the bridge and will bind the tracer with a greater affinity than the steroid alone. Consequently, the amount of native antigen required to displace the radioiodinated tracer is so great that the standard curve is shifted to the right, the slope is extremely shallow, and a loss in sensitivity and precision results. In fact, assays using iodinated labels in which a bridge homologous to that in the immunogen is used are frequently much less sensitive than their tritiated counterparts (1-5).

Several methods have been used to circumvent bridge-binding. Painter and Niswender (1,2) have shown in an estradiol-17β assay that non-equilibrium assay conditions greatly increase sensitivity using a homologous label. However, this method employs a very short incubation (5-15 min) of antibody and label which could lead to technical problems. England *et al* (7) developed a RIA for estradiol-17β that employed antibody raised against 11β-hydroxyestradiol-17β 11-succinyl-bovine serum albumin (BSA) and 11α-hydroxyestradiol-17β 11-succinyl-^{125}I-iodotyrosine methyl ester label. The heterology resulting from the stereochemical differences between the α and β linkages was such that the antibody did not recognize the succinate bridge of the trace. Unfortunately, this approach is not applicable for all steroid systems.

For example, we have found that anti-11α-hydroxyprogesterone 11-succinyl-BSA would not recognize an iodinated derivative using an 11β-bridge (8). Allen and Redshaw (5) introduced heterology by changing the chemical structure of the bridge in their labels. Unfortunately, they were not able to accomplish one of the goals for using an iodinated trace, namely increasing the assay sensitivity relative to that possible through the use of the corresponding tritiated tracer. Niswender (2) recently reported on a RIA system for progesterone using a heterologous radioiodinated trace that alleviated bridge-binding and was more sensitive than an assay using tritiated progesterone. However, the reported 50% points are extremely high when compared to other assays (3,5).

We wish to report a means of reducing antibody recognition of the tracer bridge by making subtle changes in the bridge adjacent to the steroid molecule. We used androstenedione (9) as a model and changed the commonly used succinyl ester linkage ($-O_2CCH_2CH_2CO_2$-steroid) to an ether functionality ($-NHCOCH_2O$-steroid). With this slight change a very sensitive RIA was obtained.

MATERIALS

Na[^{125}I] (carrier free) was obtained from New England Nuclear. [1,2,6,7-^{3}H]-4-androstene-3,17-dione (99 Ci/mmol) from Amersham was repurified on Sephadex LH-20 by elution with cyclohexane/benzene-/methanol (80/15/5). 19-Hydroxyandrostenedione was a gift from Searle Laboratories, Skokie, Ill. Ethyl chloroformate, ethyl diazoacetate, p-hydroxyphenylacetic acid and tyramine were purchased from Aldrich Chemical Co. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, dehydroepiandrosterone, testosterone and 5α-dihydrotestosterone were obtained from Sigma Chemical Co. Chloramine-T and sodium metabisulfite were from J.T. Baker Co. Androstenedione, androsterone, 5α-androstanedione, progesterone and estradiol-17β were purchased from Steraloids Inc., Wilton, N.H. Sephadex G-25 (fine mesh) and LH-20 were obtained from Pharmacia Fine Chemical. Pigskin gelatin was from Eastman Chemical, Merthiolate (Thimerosal) from Ruger Chemical Co., Irvington, N.J. and $RhCl_3 \cdot 3H_2O$ was from Ventron Corporation. ACS scintillation fluid was

obtained from Amersham. Solvents used were analytical grade. Steroids used as RIA standards and crossreactants were recrystalized.

SYNTHESES

The syntheses of conjugates and radioiodinated labels used in this study were prepared as 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.

19-Hydroxy-4-androstene-3,17-dione 19-hemisuccinate (II). The procedure of Bermudez et al (10) was used. A mixture of 19-hydroxyandrostenedione (1.0 gm, 3.31 mmol), succinic anhydride (1.0 gm, 10.0 mmol) and dry pyridine (10 ml) was stirred at room temperature in the dark for 7 days. The mixture was poured into 1 N HCl (150 ml) and the pH adjusted to 1.0 with concentrated HCl. The aqueous solution was then extracted five times with ethyl acetate (50 ml). The combined organic phase was dried over anhydrous Na_2SO_4. Evaporation of the solvent in vacuo yielded a clear oil (1.2 gm, 94%). Spectral data agreed with that reported previously (10). UV (3% N,N-dimethylformamide (DMF)/H_2O), λ max 245 nm (ε=12,700): IR ($CHCl_3$), νmax 3450, 1742 and 1670 cm^{-1}: NMR, p.p.m. (CD_3COCD_3), 0.92 (s,3H,18-CH_3), 2.61 (s,4H,succinyl methylene-H), 4.31 and 4.76 (dd J=12.0 cps,2H,19-CH_2O-), 5.92 (s,1H,4-H), 8.00 (broad s,1H,-CO_2H, exchanged with D_2O).

19-Hydroxy-4-androstene-3,17-dione 19-succinyl-bovine serum albumin (III). II (100 mg, 0.25 mmol) was dissolved in DMF (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 (0.02 M, pH 7.8, 20 ml) was added dropwise to the steroid solution and stirred 4 days at room temperature in the dark. The solution was dialyzed against 0.05 M $NaHCO_3$ (2 x 2000 ml) and H_2O (4 x 2000 ml) and subsequently dried by lyophilization. The molar ratio of the steroid to protein was found to be 9.2 by spectral analysis (11).

p-Acetoxyphenylacetic anhydride (IV). p-Hydroxyphenylacetic acid (36.0 gm, 0.24 mol) was dissolved in acetic anhydride (130 gm, 1.26 mol) and concentrated H_2SO_4 (0.5 ml) added. The mixture was stirred overnight at room temperature and poured into H_2O (800 ml). The solution was made basic with 1 N NaOH and extracted five times with $CHCl_3$ (200 ml). The combined organic layer was dried with Na_2SO_4 and the solvent removed in vacuo yielding a white solid. Recrystalization from benzene gave p-acetoxyphenylacetic acid as white needles (34.7 gm, 76%) mp 110-111°C. IR (CH_2Cl_2), νmax 3500, 1765, 1720 and 1512 cm^{-1}.

p-Acetoxyphenylacetic acid (10.0 gm, 51.5 mmol) was dissolved in benzene (150 ml) and refluxed for 2 hr with a Dean-Stark apparatus to

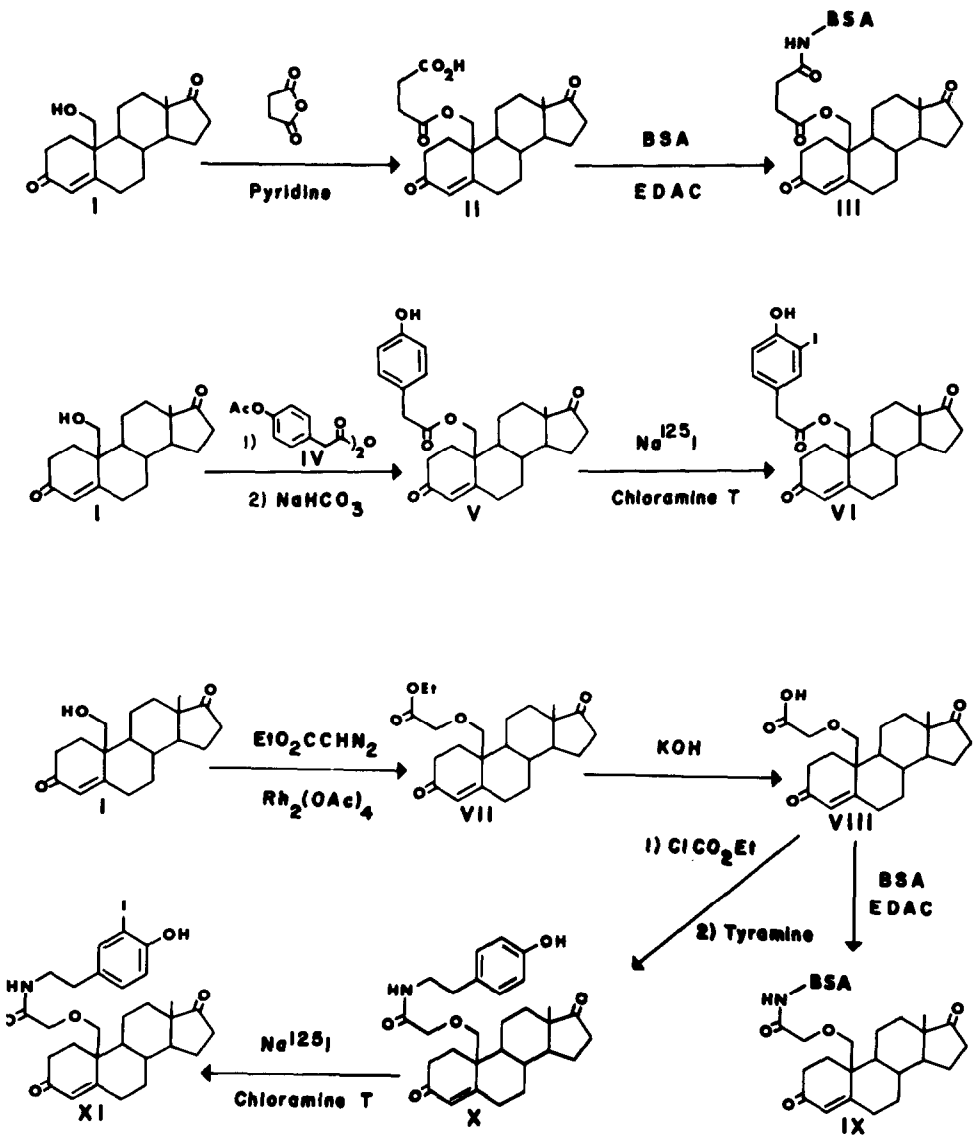

Figure 1. Synthetic Schemes.

remove H_2O. Thionyl chloride (2.2 ml) in benzene (30 ml) was added followed by the dropwise addition of pyridine (4.8 ml) in benzene (30 ml). The resulting mixture was refluxed an additional 30 min, cooled to room temperature and poured into concentrated HCl (200 ml). Water (300 ml) was added and the benzene layer removed, washed with H_2O, 1 M $NaHCO_3$ and H_2O, and dried over Na_2SO_4. Removal of the solvent in vacuo yielded

a yellow solid. Recrystalization from benzene gave IV as white needles (6.9 gm, 73%) mp 95-96°C. IR (CH_2Cl_2), νmax 1827, 1765 and 1512 cm^{-1}: NMR, p.p.m. ($CDCl_3$), 2.28 (s,6H,-CH_3CO_2-), 3.28 (s,4H,phenyl-CH_2CO-), 7.18 (m,8H,phenyl-H). Anal. Calc. $C_{20}H_{18}O_7$: C, 64.86; H, 4.90. Found: C, 65.07; H, 5.07.

19-(p-Hydroxyphenylacetoxy)-4-androstene-3,17-dione (V). 19-Hydroxyandrostenedione (1.0 gm, 3.31 mmol) and IV (2.0 gm, 5.40 mmol) were dissolved in dry pyridine (50 ml) and stirred in the dark at room temperature for 24 hr. The mixture was made acidic with 1 N HCl and extracted 5 times with $CHCl_3$ (1000 ml). The combined organic layer was then washed with 100 ml H_2O, 1 N NaOH, H_2O and 1 N HCl. The $CHCl_3$ was then dried over Na_2SO_4 and evaporated in vacuo yielding 19-(p-acetoxyphenylacetoxy)-androstenedione as a yellow oil (1.55 gm, 98%). IR (CH_2Cl_2), νmax 1740, 1671, and 1511 cm^{-1}: NMR p.p.m. ($CDCl_3$), 0.88 (s, 3H,18-CH_3), 2.28 (s,3H,CH_3CO_2-), 3.60 (s,2H,phenyl-CH_2CO-), 4.20 and 4.71 (dd J=12.0 cps,2H,19-CH_2O-), 5.86 (s,1H,4-H), 7.17 (m,4H,phenyl-H).

19-(p-Acetoxyphenylacetoxy)-androstenedione (1.0 gm, 2.09 mmol) was dissolved in methanol (30 ml). Saturated aqueous $NaHCO_3$ solution (1 ml) was added and stirred for 12 hr at room temperature. The pH of the reaction mixture was adjusted to 2.0 with 0.5 N HCl and extracted five times with $CHCl_3$. The chloroform layer was washed twice with H_2O and dried. Removal of the solvent left a yellow oil that was purified on a 3.8 x 45 cm silica gel column. Elution with benzene/ethyl acetate (4/1) yielded a clear oil. Recrystalization from acetone/hexane produced V as a white solid (514 mg, 56%) mp 143.5-144°C. IR (CH_2Cl_2), νmax 3590, 1740, 1670 and 1520 cm^{-1}: NMR, p.p.m. ($CDCl_3$), 0.85 (s,3H,18-CH_3), 3.50 (s,2H,phenyl-CH_2CO-), 4.15 and 4.65 (dd J=12.0 cps,2H,19-CH_2O-), 5.85 (s,1H,4-H), 6.85 and 7.05 (dd J=10.0 cps,4H,phenyl-H), 7.75 (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, 74.50; H, 7.56.

Radioiodination of V. V (2.0 µg, 4.58 nmol) in CH_3OH (2 µl) was placed in a 0.5 dram vial fitted with a multi-dose septum. Phosphate buffer (0.5 M, pH 7.4, 40 µl) and aqueous Na[^{125}I] (4.0 mCi, 9.0 µl) was added. The iodination was initiated by the addition of chloramine-T (20 µg, 71.0 nmol) in phosphate buffer (0.05 M, pH 7.4, 20 µl) (buffer A). After shaking at ambient temperature for 2 min the reaction was quenched by the addition of sodium metabisulfite (20 µg, 105 nmol) in buffer A (20 µl). The reaction mixture was transfered to a 0.9 x 22 cm Sephadex G-25 (fine mesh) column and eluted with buffer A to yield VI. The specific activity of the label was found to be 3.28 mCi/µg using the method of Diekman et al (12). The maximal binding of the label (active fraction) in the presence of excess antibody was 99%.

19-(Ethylcarboxymethoxyl)-4-androstene-3,17-dione (VII). 19-Hydroxyandrostenedione (100 mg, 0.33 mmol) was dissolved in dry benzene (10 ml). The catalyst $Rh_2(O_2CCH_3)_4$ (1 mg), prepared from $RhCl_3.3H_2O$ (13),

was added and the system flushed with dry N_2. Ethyl diazoacetate (1.09 gm, 9.51 mmol) was added dropwise to the steroid solution at 10°C. The reaction mixture was stirred for an additional 30 min and poured into H_2O (100 ml). The aqueous layer was extracted five times with $CHCl_3$. The combined organic layer was dried with Na_2SO_4 and evaporated *in vacuo* resulting in a yellow oil. Further purification on a 2 x 22 cm silica gel column eluted with a 0 to 2% CH_3OH gradient in $CHCl_3$ yielded VII as a clear oil (31 mg, 24%). UV (3% DMF/H_2O), λmax 248 nm (ε=13,400): IR ($CHCl_3$), νmax 1740 and 1668 cm^{-1}: NMR, p.p.m. ($CDCl_3$), 0.90 (s,3H, 18-CH_3), 1.25 (t J=7.0 cps,3H,-OCH_2CH_3), 3.88 (q J=7.0 cps,2H, -OCH_2CH_3), 4.03 (s,2H,-O_2CCH_2O-), 4.10 and 4.45 (dd J=10.0 cps,2H, 19-CH_2O-), 5.90 (s,1H,4-H). Anal. Calc. $C_{23}H_{32}O_5$: C,71.11; H,8.30. Found: C, 71.09; H,8.31.

19-(Carboxymethoxyl)-4-androstene-3,17-dione (VIII). VII (150 mg, 0.38 mmol) was dissolved in CH_3OH (10 ml). KOH (0.12 N, 6.4 ml, 0.77 mmol) was added dropwise at 10°C. The reaction mixture was then poured into saturated NaCl solution (200 ml) and made acidic with 1 N HCl. This solution was extracted six times with $CHCl_3$ (10 ml). The organic phase was dried as above and evaporated *in vacuo* yielding a clear oil (131 mg, 94%). IR ($CHCl_3$), νmax 3400, 1738 and 1670 cm^{-1}: NMR, p.p.m. (CD_3COCD_3), 0.80 (s,3H,18-CH_3), 3.75 and 3.95 (dd J=9.5 cps,2H, 19-CH_2O-), 4.00 (s,2H,-O_2CCH_2O-), 5.68 (s,1H,4-H). 7.53 (broad s,1H, -CO_2H,exchanged with D_2O).

19-[N-(Bovine serum albumin)-amidocarboxymethoxyl]-4-androstene-3,17-dione (IX). VIII (100 mg, 0.28 mmol) was reacted with EDAC and BSA as detailed above for the formation of III. The molar ratio of steroid to protein was found to be 19.1 by UV spectral analysis (11).

19-[N-[2-(p-hydroxyphenyl)-ethyl]amidocarboxymethoxyl]-4-androstene-3,17-dione (X). VIII (50 mg, 0.28 mmol) and triethylamine (28 mg, 0.28 mmol) were dissolved in freshly distilled, dry tetrahydrofuran (THF) (10 ml) and the mixture cooled to 2°C. Ethyl chloroformate (16.4 mg, 0.16 mmol) was added and stirred for 20 min. Tyramine (19.2 mg, 0.14 mmol) dissolved in THF/H_2O (3/1) (15 ml) was added to the steroid solution and the pH of the reaction mixture adjusted to 8.5 with 1 N NaOH. After 16 hr the reaction mixture was poured into H_2O (50 ml) and extracted five times with $CHCl_3$. After drying with Na_2SO_4 and removal of solvent *in vacuo* a yellow oil resulted. Final purification using a 1.3 x 35 cm silica gel column eluted with a 0 to 2% CH_3OH gradient in $CHCl_3$ yielded X as an oil (45 mg, 68%). IR ($CHCl_3$), νmax 3430 (amide N-H stretch), 3320, 1740 and 1670 cm^{-1}: NMR, p.p.m. ($CDCl_3$), 0.90 (s,3H,18,-CH_3), 2.81 (t J=6.5 cps,2H,-$NHCH_2CH_2$-phenyl), 3.70 (m,6H,-$NHCH_2CH_2$-phenyl,-$NHCOCH_2O$-, and 19-CH_2O-), 5.88 (s,1H,4-H), 6.25 (s,1H,-NHCO-), 7.08 and 7.28 (dd J=9.0 cps,4H,phenyl-H), 8.50 (broad s,1H,phenyl-OH, exchanged with D_2O). Anal. Calc. $C_{29}H_{37}O_5N$: C, 72.62; H, 7.78. Found: C, 72.56; H, 7.99.

Radioiodination of X. X (2.0 μg, 3.88 nmol) was radioiodinated with the same procedure used to produce VI. The specific activity of XI was found to be 2.91 mCi/ μg and the active fraction 97%.

METHODS

Antibodies. Antibodies against the ester-linked conjugate (III; A_4-ester-BSA) and the ether-linked conjugate (IX; A_4-ether-BSA) were raised in Suffolk ewes. Six animals were immunized with each immunogen. 5.0 mg of conjugate in Freund's complete adjuvant (1.2 ml) was injected intradermally at multiple sites on the flanks of each animal. Booster injections of 2.0 mg in incomplete Freund's adjuvant (1.2 ml) were given at weeks 4, 8 and 12. Bleedings were taken at weeks 10, 11, 14 and 15. Serum was prepared by centrifugation and stored at -20°C. The highest titers (dilution of serum required to bind 30% of the label) were found at week 11. Serum from the animal with the highest titer within each group at week 11 was used for the study.

Radioimmunoassay. The assay buffer used was 0.01 M sodium phosphate, 0.14 M NaCl, 0.01% sodium merthiolate and 0.1% pigskin gelatin, pH 7.4 (0.1% gel-PBS). Standard androstenedione (250 to 1 pg/tube) or cross-reactant steroids (1 μg to 12.5 pg/tube) dissolved in assay buffer was added to duplicate tubes as described previously (7). Antibody (200 μl)(anti-A_4-ester-BSA; 1/100,000 dilution for use with iodinated labels or 1/10,000 dilution with tritiated androstenedione and anti-A_4-ether-BSA; 1/50,000 dilution for use with iodinated traces or 1/4,000 dilution with tritium) in 0.1% gel-PBS + 0.05 M EDTA was added. Finally, label (100 μl)([^{3}H]-androstenedione (A_4-^{3}H), 34 pg/16,000 cpm; VI (A_4-ester-L), 6.8 pg/40,000 cpm; or XI (A_4-ether-L), 7.5 pg/40,000 cpm) diluted in assay buffer was added. Triplicate total count, background and buffer control tubes contained 100 μl label, 100 μl label plus 700 μl buffer or 100 μl label, 200 μl antibody plus 500 μl buffer respectively. Crossreactivity studies were done using the method of Thornycroft et al (14). The assay tubes were vortexed and incubated at room temperature for 2 hr. Charcoal solution (1 ml)(0.25% charcoal and 0.025% dextran in PBS buffer) was added for separation of bound label from free. The tubes were vortexed, incubated for 10 min at 4°C and centifuged at 800 x g for 15 min. The supernatants and total count tubes for the assays using iodinated labels were counted for 1 min in a Searle 1185 gamma counter. When tritiated label was employed the supernatants were added to scintillation fluid (10 ml) and counted for 4 min in a Searle Isocap beta counter. Results were analyzed by computer as described elsewhere (15).

RESULTS AND DISCUSSION

Figure 1 illustrates the structures of the conjugates and radioiodinated labels used in this study. Although the ester and ether bridges are only slightly different (the ester carbonyl group is one carbon atom farther from the steroid molecule than in the ether linkage), the difference was sufficient to reduce bridge-binding. Standard curves were obtained using the antibodies against the ester-linked (anti-A_4-ester-BSA) or ether-linked (anti-A_4-ether-BSA) conjugates and measuring the amount of bound label (A_4-ester-L, A_4-ether-L or tritiated androstenedione, A_4-^3H) in the presence of varying amounts of androstenedione. The curves were linear when logit-log transformation was employed. The total amount of label bound (no unlabeled steroid), the amount of unlabeled steroid needed to displace 50% of the bound trace (50% point) and the slope of the standard curves are shown in table 1.

The most striking difference is the change in assay sensitivity when the homologous and heterologous radioiodinated labels are compared. Anti-A_4-ester-BSA used with the heterologous A_4-ether-L gave a 50% point much lower (15 pg vs. 625 pg) than that obtained with the homologous A_4-ester-L. As expected the total binding of the homologous label was greater (30.3% vs 65.0%). This trend was reversed when anti-A_4-ether-BSA was used, (425 pg vs. 76 pg and 62.4% binding vs. 33.4%). We use the 50% inhibition point for comparison in interassay sensitivies because it is much more stable and provides an accurate comparison of curve location. However, the actual limits of detection (16) were shown to be 150-250 femtograms for the A_4-ether-L/anti-A_4-ester-BSA combination and 0.9-1.1 pg for A_4-ester-L/anti-A_4-ether-BSA.

Table 1

Antibody	Dilution	Label	Binding	50% Point	Slope
Anti-A_4-ester-BSA	1/100,000[a]	A_4-ether-L[b]	30.3%	15 pg/tube	-1.64
"	1/100,000	A_4-ester-L[c]	65.0%	625 "	-0.72
"	1/10,000[d]	A_4-^3H[e]	32.3%	99 "	-2.32
Anti-A_4-ether-BSA	1/50,000[f]	A_4-ether-L	62.4%	425 "	-0.87
"	1/50,000	A_4-ester-L	33.4%	76 "	-1.99
"	1/4,000[g]	A_4-^3H	31.7%	128 "	-2.39

[a]Final dilution; 1/400,000
[b]40,000 cpm; 7.5 pg/tube
[c]40,000 cpm; 6.8 pg/tube
[d]Final dilution; 1/40,000
[e]16,000 cpm; 34 pg/tube
[f]Final dilution; 1/200,000
[g]Final dilution; 1/16,000

Clearly the use of a heterologous bridge drastically decreases bridge-binding and increases sensitivity. Another important advantage of using a heterologous label is the increased slope of the standard curve. As the magnitude of the slope approaches the value obtained using a tritiated tracer it indicates that the affinity of the antibody for the tracer and the native steroid are more nearly similar and relatively less native antigen is required to inhibit binding of the label. The steeper slope yields a more precise assay because less mass of standard is required to elicit an equivalent change in % binding and the percent of radiolabeled tracer bound to the antibody can be increased without unacceptable losses of sensitivity. This study also demonstrates that the use of a heterologous trace can yield an assay that is more sensitive than one in which tritiated labels are employed (50% point of 15 pg vs. 99 pg for anti-A_4-ester-BSA and 76 pg vs. 128 pg for anti-A_4-ether-BSA). An additional advantage is the higher dilution of antibody used in assays employing radioiodinated traces. In both

cases the antibody dilution used with the radioiodinated label was at least 10-fold greater than that used with tritium. Crossreactivities of both antisera with other steroids are shown in table 2.

Table 2

	%Crossreactivity	
Steroid	Anti-A_4-ester-BSA	Anti-A_4-ether-BSA
Androstenedione	100.0	100.0
5α-Androstanedione	52.2	61.7
19-Hydroxyandrostenedione	25.3	26.5
Testosterone	0.05	0.3
5α-Dihydrotestosterone	0.04	0.1
Dehydroepiandrosterone	26.0	10.0
Androsterone	48.1	34.8
Progesterone	0.03	0.1
Estradiol-17β	0.1	0.08

The crossreactivity of 5α-androstanedione was not unexpected. Antibodies apparently can not differentiate between the 4-ene and 5α-reduced forms of androgens (10,17-19). Likewise, the crossreactivity of the 19-hydroxyandrostenedione was expected since the conjugates were made from this compound (19). However, in addition to the problem of recognizing 5α-androstanedione and the 19-hydroxy compound the antibodies bound dehydroepiandrosterone and androsterone. These steroids possess 3β-hydroxy-5-ene and 3α-hydroxy-5α-dihydro functionalities respectively which are quite different from the 3-oxo-4-ene configuration of androstenedione. Apparently when androstenedione is conjugated to the carrier protein through the 19 position, the A-ring of the steroid, in particular the 3 position, may be partially enveloped by the carrier protein and consequently the antibodies do not distinguish between 3-oxo or 3-hydroxy steroids. This phenomenon has been seen recently for other 19-ester (10) or 19-ether

linked (20) androstenedione conjugates. In spite of the crossreactivities with the 5α-reduced and 3-hydroxy steroids the 19-androstenedione system is a good model for demonstrating the existence of bridge-binding and a method for reducing it. We have shown that through the use of a heterologous radioiodinated label a very precise and sensitive assay can be obtained, and in addition, the sensitivity is greater than in assays that utilize tritiated traces.

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