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SYNTHESIS OF NEW STEROID HAPTENS FOR RADIOIMMUNOASSAY—PART V. 19-*O*-CARBOXYMETHYL ETHER DERIVATIVE OF TESTOSTERONE. A HIGHLY SPECIFIC ANTISERUM FOR IMMUNOASSAY OF TESTOSTERONE FROM BOTH MALE AND FEMALE PLASMA WITHOUT CHROMATOGRAPHY*

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SUMMARY

Synthesis of the 19-*O*-carboxymethyl ether derivative of testosterone and the preparation of its bovine serum albumin (BSA) conjugate is described. By employing this conjugate, antiserum was raised in rabbits which proved to be specific for testosterone. A radioimmunoassay (RIA) procedure is described for measurement of testosterone from both male and female plasma after ether–chloroform (4:1) extraction of the sample without prior chromatography.

INTRODUCTION

We have recently descibed [1] a 15β-linked anti-testosterone serum, exhibiting less than 2% cross-reaction with 5α-dihydrotestosterone, that can be employed for the measurement of testosterone from male plasma samples without prior chromatography. However, its minor cross-reaction with progesterone and other related C-21 steroids presented a serious problem for the determination of testosterone in human female plasma samples. We have synthesized a new steroid hapten and covalently coupled it to bovine serum albumin (BSA) to give a conjugate which generated a very specific anti-testosterone serum. It is now possible to measure testosterone levels in both male and female plasma extracts without chromatographic purification. In this communication we present details concerning the synthesis of the steroid hapten (Fig. 1), the 19-*O*-carboxymethyl ether derivative of testosterone (**7**), the preparation of its BSA conjugate, the production of anti-testosterone serum, and its application in the radioimmunoassay (RIA) of plasma testosterone.

* The methods described herein are the subject of a pending United States patent application.

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EXPERIMENTAL

Solvents and reagents

All solvents, reagents, and unlabeled steroids were purchased and purified as previously reported [1]. *N,N'*-Carbonyldiimidazole was obtained from Aldrich Chemical Co., Inc. [1,2,6,7-^{3}H]-Testosterone, 89–110 Ci/mmol, was purchased from Amersham Searle Corp. Mass spectral data were generated with a Finnigan 1015 F-L quadrapole mass spectrometer. n.m.r. Spectra were obtained with a Varian-EM 390 spectrometer in deuterochloroform using tetramethylsilane (TMS) as the internal standard and are reported in ppm. All other spectra were obtained as described earlier [1]. Dry column chromatography was performed on Woelm silica gel in a nylon column as described by Loev and Goodman[2]. The microanalyses were performed by either Micro-Tech Laboratories, Skokie, IL, or Midwest Microlab, Inc., Indianapolis, IN.

Synthesis of the steroid hapten

19-Hydroxyandrost-4-ene-3,17-dione (**1**), obtained from Syntex, served as the starting material. The 19-hydroxyl group in (**1**) was first protected by reacting (**1**) with dihydropyran in the presence of *p*-toluenesulfonic acid in dioxane [3] to give the tetrahydropyranyl ether (THP) (**2**). Reduction of (**2**)

with diisobutylaluminum hydride (DIBAL), followed by *in situ* Oppenauer oxidation of the aluminum alkoxide with isopropanol-acetone as described by Eder[4], gave 17β-hydroxy derivative (**3**) in excellent yield. The 17β-hydroxyl group in (**3**) was then protected as an acetate by reacting it with acetic anhydride in pyridine solution to give (**4**). Subsequent cleavage of the THP ether using 3:1:1 acetic acid–tetrahydrofuran–water at 70°C [5] gave the 19-hydroxytestosterone 17β-acetate (**5**), which was earlier prepared by Pfenninger *et al.*[6] by a different method. The 19-hydroxy compound (**5**) was reacted with ethyl diazoacetate in benzene solution in the presence of rhodium tetraacetate [7] to give in low yield the 19-ethoxycarbonylmethyl ether derivative (**6**). Hydrolysis of (**6**) with sodium methoxide in methanol gave the 19-O-carboxymethyl ether derivative (**7**).

The following sequence describes the typical procedure employed in the workup and isolation of the reaction product. The reaction mixture was treated with ice-water, and then extracted with a specified organic solvent. The organic extract was washed with brine, dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated under reduced pressure on a rotary evaporator at 60–65°C. The residue remaining in the flask was then purified as described.

Androst-4-ene-3,7-dione-19-(2′-tetrahydropyranyl) ether (**2**). Freshly distilled dihydropyran (5 ml) was added dropwise over 3 min to a solution of 19-hydroxy 4-androstene-3,17-dione (5 g) and *p*-toluene-sulfonic acid (250 mg) in anhydrous dioxane (50 ml). The reaction was stirred for 3 additional min and then was brought to pH 8 with half-saturated methanolic ammonia. The dioxane was evaporated under vacuum and the reaction product was isolated with chloroform. The tetrahydropyranyl ether **2** (5.4 g, 82%) crystallized from ether–petroleum ether to give the analytical sample m.p. 140–142°; ν_{Max} 1740, 1665, 1620 cm^{-1}; $\delta(CDCl_3)$: 0.92 (*s*, 18-CH_3), 3.57 and 4.16 (d, J = 9 Hz, 19-CH_2), 4.61 (*m*, 2′-H), 5.93 (*s*, 4-H) ppm; MS, *m/e* = 386 (M^+). Anal. Calc. for $C_{24}H_{34}O_4$: C, 74.58; H, 8.87. Found: C, 74.55; H, 8.95.

3-oxo-17β-acetoxyandrost-4-en-19-yl 2′-tetrahydropyranyl ether (**4**). A solution of diisobutylaluminum hydride in tolene (24.8%, 16.4 ml) was added through a rubber septum over 8 min to a stirred, cold (0°C) solution of **2** (3.7 g) in toluene (80 ml). The reaction mixture was stirred under nitrogen at 0°C for 1 h and then maintained at room temperature for an additional h. After cooling at 0°C once again, acetone (5.3 ml) and *iso*propanol (5.3 ml) were added slowly and then the reaction was stirred at room temperature for 14 h. The reaction mixture was acidified with 1N sulfuric acid and then immediately neutralized with solid sodium bicarbonate to prevent the hydrolysis of THP ether. The gelatinous ppt of aluminum carbonate was filtered and the product was isolated from the filtrate with ethyl acetate. 3-oxo-17β-hydroxy 4-androsten-19-yl 2′-tetrahydropyranyl ether **3** (5.6 g) was obtained as a gum and resisted crystallization. Its purity was established by thin layer chromatography. ν_{Max} 3440, 1668, 1622 cm^{-1}; $\delta(CDCl_3)$; 0.78 (*s*, 18-CH_3), 3.57 and 4.17 (*d*, *J* = 9 Hz, 19-CH_2), 4.58 (*m*, 2′-H), 5.90 (*s*, 4-H) ppm.

The 17β-hydroxy compound **3** (5.6 g) was dissolved in pyridine (35 ml), and acetic anhydride (35 ml) was added and the mixture set aside at room temperature in the dark for 18 h. The reaction mixture was then evaporated under vacuum and the residue was purified on a dry silica gel column (32 × 790 mm) employing ether–ethyl acetate (8:2, v/v) as the developing solvent. The silica gel column, from 500–690 mm from origin was cut and extracted with

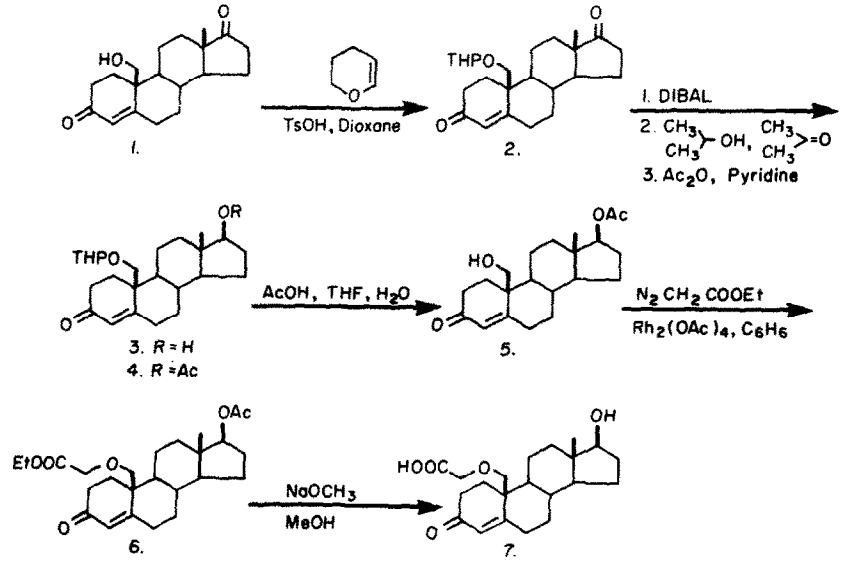

Fig. 1. Synthesis of the steroid hapten. TSOH = *p*-Toluenesulfonic acid, DIBAL = Diisobutylaluminum hydride, THP = Tetrahydropyranyl, THF = Tetrahydrofuran.

ethyl acetate to give pure tetrahydropyranyl ether **4** (2.29 g, 53%) which on crystallization from ether–petroleum ether melted at 128–129°C. ν_{Max} 1740, 1675, 1625, 1250 cm^{-1}; $\delta(CDCl_3)$: 0.83 (*s*, 18-CH_3), 2.02 (*s*, 17-OAc), 3.55 and 4.14 (*d*, J = 9 Hz, 19-CH_2), 4.61 (*m*, 2′-H, and 17-H), 5.80 (*s*, 4-H) ppm; MS, m/e = 430 (M^+). Anal. Calc. for $C_{26}H_{38}O_5$: C, 72.53; H, 8.90. Found: C, 72.49; H, 8.98.

3-oxo-19-hydroxy 4-androsten-17-yl acetate (**5**). The THP ether **4** (2.3 g) was dissolved in a mixture of acetic acid (45 ml), water (15 ml), and tetrahydrofuran (15 ml) and stirred at 70°C for 3 h. The reaction mixture was then cooled and evaporated to dryness under reduced pressure. The product so obtained was combined with the residue obtained by similar hydrolysis of **4** (0.3 g) from an earlier batch. The amount from both batches was combined and crystallized from methylene chloride–ether to give **5** (1.57 g) m.p. 166–168°C; ν_{Max} 3350, 1745, 1667, 1620, 1240 cm^{-1}; λ_{Max} 243 nm (ϵ = 15,116) (MeOH); $\delta(CDCl_3)$ 0.82 (*s*, 18-CH_3), 2.02 (*s*, 17-OAc), 3.91 and 4.10 (*d*, J = 10.5 Hz, 19-CH_2), 4.65 (*m*, 17-H), 5.95 (*s*, 4-H) ppm; MS, m/e = 346 (M^+) Lit. [6] m.p. 162–163°. Anal. Calc. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.92; H, 9.04.

3-oxo-17β-hydroxyandrost-4-en-19-yl carboxymethyl ether (**7**). To a solution of the 19-alcohol **5** (200 mg) and rhodium (II) acetate dimer (2 mg) in benzene (8 ml), ethyldiazoacetate (2 ml) in benzene (3 ml) was added dropwise over a period of 40 min. The mixture was then stirred at room temperature for 16 h. The rhodium catalyst was removed from the solution by filtration through silica gel. The silica gel was washed with ethyl acetate and the organic solvent evaporated to give the desired product (**6**) contaminated with unreacted starting material. Another 200 mg batch was processed similarly and both batches were combined and purified on a dry silica gel column (15 × 1090 mm) employing ether as the developing solvent. The silica gel column, from 745 to 870 mm from the origin, was cut and extracted with ethyl acetate to give 122 mg of the ethyl ester (**6**) as an oil (24%). ν_{max} 1738, 1665, 1623, 1250 cm^{-1}; $\delta(CDCl_3)$: 0.84 (*s*, 18-CH_3), 1.30 (*t*, J = 7 Hz, –O–CH_2–CH_3), 2.00 (*s*, 17-acetate), 3.73 and 3.95 (*d*, J = 9 Hz, 19-CH_2), 4.03 (*s*, –O–CH_2–C(=O)–OET), 4.20 (*q*, J = 7 Hz, –O–CH_2 CH_3), 4.53 (*m*, 17-H), 5.90 (*s*, 4-H) ppm.

The recovered starting material **5** (0.269 g) was recycled.

The 19-ether **6** (132 mg) obtained from different batches, was dissolved in anhydrous methanol (10 ml), and sodium methoxide (200 mg) was added and stirred at room temperature under nitrogen. After 45 min, the methanol was evaporated under nitrogen and the residue was diluted with water. Unhydrolyzed material was removed by extraction with methylene chloride. The aq. solution was cooled in an ice bath, then acidified to pH 2 with conc. HCl. The acid (**7**) was isolated with methylene chloride and crystallized from methylene chloride-ether (41 mg, 37%), m.p. 190–192°C; ν_{max} 3430, 1740, 1655 cm^{-1}; λ_{max} 242 (ϵ = 13,449); $\delta(CD_3OD)$ 0.78 (s, 18-CH_3), 3.57 (*m*, 17-H), 3.80 and 4.02 (*d*, J = 9 Hz, 19-CH_2), 4.04 (*s*, –O–CH_2–C(=O)–OH), 5.84 (*s*, 4-H) ppm; MS, m/e = 362 (M^+). Anal. Calcd. for $C_{21}H_{30}O_5$: C, 69.59; H, 8.34. Found: C, 69.40; H, 8.26.

Preparation of the steroid–bovine serum albumin conjugate and determination of the moles of steroid bound per mole protein

3-Oxo-17β-hydroxyandrost-4-en-19-yl carboxymethyl ether (**7**) was coupled to BSA by a mild procedure developed by Axen[8] using *N,N′*-carbonyldiimidazole. The steroid hapten (0.127 mmol) was dissolved in 5 ml of anhydrous dimethylformamide (DMF), and *N,N′*-carbonyldiimidazole (0.254 mmol) was added and the mixture stirred under nitrogen for 20 min. BSA equivalent to 0.127 mmol of available amino groups was dissolved in a 1:1 mixture of water and DMF (30 ml), and the pH was adjusted to 9.5 with triethylamine (TEA). The DMF solution containing the imidazolide was slowly added to the well-stirred aq. DMF solution of BSA. Water (5 ml) was added to the reaction mixture and the pH readjusted to 10.5 with TEA. The reaction was allowed to proceed at room temperature overnight with stirring. The solution was then dialyzed for 24 h at 4°C against a constant flow of distilled water. The dialysate was dried to a fluffy white powder by lyophilization to give 176 mg of the conjugate (90% yield). U.V. absorption analysis [9] and determination of the free amino groups in the conjugate by a quantitative ninhydrin procedure [10] showed that the number of moles of steroid bound per mole of protein was 27 and 22, respectively.

Immunization procedure and collection of the antibody

Four female New Zealand white rabbits, 4 months old, were used for immunization. The injection and bleeding schedules were exactly as reported previously [1].

Assay procedure

Standards were prepared from stock solutions of unlabeled steroids in absolute ethanol (100 ng/ml). Two working standard solutions, containing 1 ng/ml and 20 ng/ml, were prepared in sodium phosphate assay buffer (0.1 M, pH 7, 0.9% NaCl). The labeled [1,2,6,7-^{3}H]-testosterone was prepared in assay buffer at a concentration of 100 pg/ml. The antiserum was prepared in BSA-assay buffer (1 g BSA/1000 ml sodium phosphate buffer) at a concentration of one-fifth of the final working dilution.

Plasma (0.3 ml from men, 1.0 ml from women), 0.3 N NaOH (1 drop/0.1 ml plasma), and distilled water (0.5 ml in male only) were added to a

16 × 125 mm tube and mixed on a Vortex mixer. [1,2,6,7-^{3}H]-Testosterone (0.2 ml, 2000 c.p.m.) was then added to correct for recovery and the sample was mixed well. Diethyl ether–chloroform (4:1, v/v) (2 ml) was added for extraction of the steroid. The extraction was repeated three times and the combined fractions were washed with distilled water (1 ml) and the solvent removed by evaporation. In the direct measurement, assay buffer (0.4 ml for men, 0.7 ml for women) was added to the sample tube and mixed, 0.1 ml was removed to count for recovery and 0.1 ml (2 ×) (men), or 0.5 ml (women) was removed for assay.

As reported previously [1] a modification of the column chromatography as described by Murphy[11] was employed in the purification of the testosterone fraction. Glass columns (880 × 10 mm) fitted with Teflon stopcocks were packed with 15 g of Sephadex LH-20 to a height of 43 cm in chloroform–heptane–ethanol 50:50:0.25. The extracted sample was transferred to the column in 2 × 0.5 ml of the same solvent mixture. The first 57 ml eluted from the column were discarded and the next 11 ml (fractions 58 through 68) were collected as the testosterone fraction in a 16 × 125 mm tube and taken to dryness under nitrogen. The tube was then rinsed with column solvent to concentrate the steroid and again taken to dryness. The sample tubes were then treated as above in the direct method.

The standard curve was established by preparing duplicate 3-ml centrifuge tubes containing 0, 50, 100, 250, 500, 1000, and 2000 pg of the steroid in a total vol. of 0.5 ml assay buffer. When column chromatography was employed, 0.5 ml of column solvent was added to each tube and evaporated to dryness prior to the addition of the steroid standard and assay buffer. For plasma from men, the sample tubes were prepared in duplicate with plasma extract (0.1 ml) and 0.4 ml assay buffer for a total volume of 0.5 ml. For plasma from women, the sample tubes were prepared with plasma extract (0.5 ml) only. To all standard and sample tubes, antibody (0.25 ml) and labeled testosterone (0.5 ml) were added. These were mixed on a Vortex mixer and allowed to incubate at 4°C overnight. After addition of 0.2 ml gamma globulin dextran-coated charcoal (1 g charcoal, 0.1 g dextran, 0.2 g human gamma globulin, 200 ml deionized water), each tube was again mixed and placed in a cold room (4°C) for 20 min. After centrifugation at 2,500 rev./min for 6 min, 0.5 ml of each supernatant was aliquoted into a counting vial. Then 15 ml of scintillation medium (4 g PPO, 50 mg dimethyl-POPOP, 50 ml BBS-3, and 1,000 ml toluene) were added to each vial. The samples were counted to a relative standard error [12] of less than 2% in a Packard model 3320 liquid scintillation counter.

RESULTS AND DISCUSSION

All four rabbits immunized with the testosterone-19-BSA ether linked conjugate produced antisera with high titers and specificity in 7 months' time. The titer was determined from the ability of antibody to bind a constant amount (50 pg) of the labeled steroid as compared to plasma collected prior to the primary injection. A preliminary evaluation of the cross-reactivity [13] of 5α-dihydrotestosterone (5α-DHT) with the antiserum obtained from each rabbit (Table 1), allowed us to select the antiserum which exhibited low cross-reactivity with sufficiently high titer and sensitivity for complete characterization. The antiserum from rabbit X-181 was used in the present studies. As reported in our earlier procedure [1], a binding affinity determination was carried out and the data were evaluated as a Scatchard plot [14]. The binding affinity constant K_a of this antiserum is 6.15×10^9 l/mol. The antibody proved to be very specific and showed 6.7% cross-reaction [13] to 5α-dihydrotestosterone. Since the hapten employed is a derivative of 19-hydroxytestosterone, the antiserum exhibits 28.6% cross-reaction with this compound. Other steroids that showed minor cross-reaction include 11-oxotestosterone (1.8%) and 5α-androstane-3β,17β-diol (2.2%). There is no mention in the literature of the presence of detectable amounts of 19-hydroxytestosterone or 11-oxotestosterone in human peripheral plasma. As a result, these compounds would not affect the measurement of testosterone by RIA. Indeed, our results with and without chromatography support the presumed absence of these compounds as the values in both cases are identical. The cross-reactivity data are presented in Table 2.

Validation of testosterone radioimmunoassay

The accuracy of the testosterone assay was assessed by determining the testosterone levels: (1) in normal pooled male and female plasma samples with and without chromatographic purification; (2) by the addition of known amount of testosterone to normal pooled male and female plasma; and (3) by the addition of a known amount of testosterone in the presence of increasing amounts of 5α-DHT in pooled male plasma. The results are presented in Tables 3, 4 and 5, respectively.

A comparison of serum testosterone levels in a normal cycling female over a period of 2 months determined with 15-linked anti-testosterone serum [1] employing chromatography and with 19-linked anti-testosterone serum without chromatography is pres-

Table 1. 5α-Dihydrotestosterone cross-reactivity with 19-linked anti-Testosterone serum

Rabbit No.	Production time	Titer	Percent cross with dihydrotestosterone
X-181	7 months	1:175,000	5.80
X-182	7 months	1:20,000	18.79
X-183	7 months	1:40,000	17.80
X-184	7 months	1:40,000	34.20

Table 2. Cross-reactivity data of 19-linked anti-testosterone serum

Steroid	% Cross-reactivity
1. Testosterone	100.00
2. 19-Hydroxytestosterone	28.67
3. 5α-Dihydrotestosterone	6.65
4. 11β-Hydroxytestosterone	0.00
5. 11-Oxotestosterone	1.80
6. 11β-Hydroxyandrostenedione	0.00
7. Androstenedione	0.90
8. 5α-Androstane-3,17-dione	0.00
9. 5β-Androstane-3,17-dione	0.00
10. 5β-Dihydrotestosterone	0.63
11. Androsterone	0.00
12. Epiandrosterone	0.19
13. 5α-Androstane-3α,17β-diol	0.00
14. 5α-Androstane-3β,17β-diol	2.19
15. 5-Androstene-3β,17β-diol	0.51
16. Dehydroepiandrosterone	0.00
17. 6β-Hydroxytestosterone	0.75
18. Estrone	0.00
19. Estradiol-17β	0.43
20. Estriol	0.00
21. Progesterone	0.00
22. Corticosterone	0.00
23. Deoxycorticosterone	0.00

ented in Table 6. The increased concentration of progesterone during the luteal phase has no effect on the measurement of testosterone with 19-linked anti-testosterone serum.

Testosterone has been coupled to carrier protein through nine different positions out of the possible nineteen carbons, to obtain antigens capable of generating specific anti-testosterone serum. Only three conjugates prepared by coupling through C-1 [15], C-11 [16], and C-15 [1] produced anti-testosterone sera exhibiting low cross-reaction with 5α-DHT; 8%, 15%, and 1.8% respectively. Although these antisera proved useful for measurement of testosterone in human male plasma by direct assay, they were unsuitable for human female plasma samples. These different antisera cross-reacted to a certain extent with other circulating steroids, such as 11-oxy-C-19 steroids [16], progesterone, corticosterone, and deoxycorticosteroid [1]. Progesterone cross-reactivity, in particular, made the C-15 antisera [1] unsuitable for use with female plasma. In each case, testosterone was grossly overestimated when the female plasma extracts were subjected to direct assay. The new antiserum obtained with testosterone 19-*O*-carboxymethyl ether conjugate

Table 3. Testosterone levels in pooled male and female plasma, with and without chromatographic separation

Sample source	Type of measurement	Testosterone measured (ng/ml)	Coefficient of variation (%)	No. of samples
Male	Extraction only	5.76	6	16*
	LH-20 Chromatography	5.77	3	16*
Female	Extraction only	0.26	4	8
	LH-20 Chromatography	0.24	7	8

* Duplicate determination.

Table 4. Recovery of testosterone added to 100 μl of pooled male and female plasma

Sample source	Testosterone added (ng)	Testosterone measured (ng)	Difference (ng)	Recovery (%)	Coefficient of variation (%)	No. of samples in triplicate
Male	0	0.57	—	—	—	8
	1	1.62	1.05	103	6	8
	2	2.63	2.05	102	8	8
	4	4.62	4.05	101	9	8
Female	0	0.03	—	—	—	8
	1	1.11	1.08	108	5	8
	2	2.09	2.06	103	8	8
	4	4.08	4.05	101	4	8

Table 5. Recovery of testosterone added to 100 μl of pooled male plasma with increasing amounts of 5α-dihydrotestosterone

Testosterone added (ng)	DHT added (ng)	Testosterone measured (ng)	Difference (ng)	Recovery (%)	Coefficient of variation (%)	No. of samples
0	0	0.850	—	—	9	6
1	0	1.829	0.979	98.9	9	6
1	0.25	1.878	1.028	101.5	5	6
1	0.50	1.830	0.980	98.9	12	6
1	1.00	1.831	0.981	98.9	7	6

Table 6. Comparison of testosterone levels determined with a 15β-linked and a 19-linked anti-testosterone serum in plasma obtained from a normal cycling female

Cycle	Cycle day	15β-Linked Anti-testosterone serum With t.l.c. (ng %)	19-Linked Anti-testosterone serum Without t.l.c. (ng %)	Progesterone* (ng %)
1st Mo.	9	37.9	37.5	13.3
	10	155.5†	32.3	19.6
	11	118.2†	33.0	20.3
	12	30.6	34.8	34.7
	13 (ovulation)	45.2	35.2	94.2
	14	22.6	22.4	126.8
	15	40.5	32.6	250.5
	16	38.6	29.2	323.6
	17	43.3	29.7	304.1
	20	74.6	23.2	472.0
	23	40.7	25.8	305.2
	27 (menses)	34.1	26.1	43.2
2nd Mo.	7	79.0	33.2	12.1
	9	64.2	37.8	17.2
	11 (ovulation)	47.8	42.3	69.9
	15	157.2†	28.0	228.5
	18	55.8	26.6	647.1
	21	44.1	24.8	510.9
	25	86.4	30.4	270.2

* Progesterone was determined by a modified RIA procedure [19]. Intra- and interassay coefficient of variation, 4 and 8% respectively. † Unexplainable higher values obtained on chromatographic samples.

proved to be specific enough so that it is now possible to quantitate testosterone levels in both male and female plasma extracts without prior chromatographic separation since values with and without chromatography were identical.

Earlier attempts to obtain specific antibodies to testosterone by employing carboxylic acid esters such as testosterone-19-hemisuccinyl-BSA [17] or testosterone-19-hemiglutaryl-BSA conjugates [18] resulted in nonspecific antisera with which 5α-DHT exhibited 50–60% cross-reaction. It is clear that the site of conjugation and also the nature of the chemical "handle" through which the steroid is coupled to carrier protein play an important role in producing specific antisera. The steroid conjugate with an ether linkage apparently presents itself in a more favorable conformation to generate antisera with high specificity. Also, a steroid hapten with an ether linkage is more stable than an ester under the alkaline conditions (pH 8–9.5) usually employed during the preparation of the steroid-protein conjugate.

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