Production of Highly Specific Anti-testosterone Antiserum by an Immunotolerance Method

Yoshinori MIYAKE, Minoru SHIGETOSHI and Masaharu KOJIMA*

Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan. *Received May 27, 1989*

Anti-testosterone antisera were produced by pretreatment of rabbits with 15α-carboxymethyl-5α-dihydrotestosterone linked to a copolymer of D-glutamic acid and D-lysine (D-GL) before immunization with the bovine serum albumin conjugate of 15α- and 15β-carboxymethyltestosterone. The specificity for 5α-dihydrotestosterone of the anti-testosterone antisera was considerably improved.

Keywords testosterone radioimmunoassay; immunotolerance method; 15α-carboxymethyltestosterone; 15β-carboxymethyltestosterone; anti-testosterone antiserum; cross-reactivity

Introduction

Since the successful production of anti-steroid antiserum by Erlanger *et al.*,[1] research has been directed towards obtaining highly specific antibodies. The specificity of an antibody is affected by the chemical form of the bridge group through which the steroid is attached to the carrier protein and by the binding site on the steroid which is attached to the carrier protein. Accordingly, it is important to choose an appropriate chemical form of the bridge group and an appropriate binding site in order to produce highly specific antiserum.

In radioimmunoassay of testosterone, anti-testosterone antiserum is required to have low or no cross-reactivity with 5α-dihydrotestosterone (5α-DHT) and C-21 steroids coexisting with testosterone in blood, because testosterone level can be grossly overestimated as a consequence of minor cross-reactions with 5α-DHT and C-21 steroids.[2] In our previous paper,[3] we obtained anti-testosterone antisera (anti-15α- and 15β-CMT antisera) with low cross-reactivity for C-21 steroids and little cross-reactivity for 5α-DHT by immunization with the bovine serum albumin (BSA) conjugate of 15α- and 15β-carboxymethyltestosterone (15α- and 15β-CMT), respectively. The result revealed that the 15-carboxymethyl group is an appropriate bridge group for production of highly specific anti-testosterone antiserum.

On the other hand, Tateishi *et al.*[4] developed an immunization procedure for production of anti-testosterone antiserum with low cross-reactivity for 5α-DHT by the method reported by Hamaoka *et al.*[5] The procedure involved the inactivation of cross-reacting antibody-forming precursor cells by pretreatment with 5α-DHT linked to a copolymer of D-glutamic acid and D-lysine (D-GL) followed by immunization with testosterone conjugated to carrier protein (pretreatment method). Namely, when 15β-carboxyethylmercaptotestosterone (15β-CET)-keyhole limpet hemocyanin conjugate as an immunogen and 15β-carboxyethylmercapto-5α-dihydrotestosterone D-GL conjugate as a tolerogen were used, anti-testosterone antiserum showed the lowest cross-reactivity (0.49%) for 5α-DHT among anti-testosterone antisera so far obtained, but somewhat higher cross-reaction with C-21 steroids such as progesterone (19.3%), deoxycorticosterone (2.8%) and corticosterone (2.9%), which presented a serious problem for determination of testosterone in female human serum. The pretreament method was further tested by Dupret *et al.*[6] with a view to improving the specificity (*i.e.*, reducing the cross-reactivity with 5α-DHT) of anti-testosterone antiserum and it was revealed that a strong immunotolerance to the hapten structure can be induced.

Accordingly, the specificities of anti-15α- and 15β-CMT antisera are expected to be enhanced by the application of the pretreatment method. The present paper deals with the specificity of anti-15α- and 15β-CMT antisera produced with 15α- and 15β-CMT–BSA conjugates by the pretreatment method.

Materials and Method

All melting points are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JNM PS 100 spectrometer, with tetramethylsilane as an internal standard. Infrared (IR) spectra were recorded on a JASCO IRA-1 spectrometer. Optical rotations were determined with a JASCO DIP-SL automatic polarimeter at 20 °C. Elemental analysis were performed by the staff of the microanalytical section of Kyushu University.

Reagents: D-Glutamic acid and D-lysine copolymer (D-GL) was as a gift from Daiichi Radioisotope Laboratories (Tokyo, Japan). 15α- and 15β-CMT–BSA conjugates[3] which had been prepared by us were used. Freund's complete adjuvant was purchased from Difco Laboratories (Detroit, MI). [1α,2α-^{3}H(n)]Testosterone, 1.8 TBq/mmol, was purchased from New England Nuclear. All unlabeled steroids were purchased from Steraloids, Inc. (Wilton, NH) and other reagents from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of 15α-Carboxymethyl-17β-hydroxy-5α-androstan-3-one (5α-DHT-15-CM) Preparation of 5α-DHT-15-CM is shown in Chart 1.

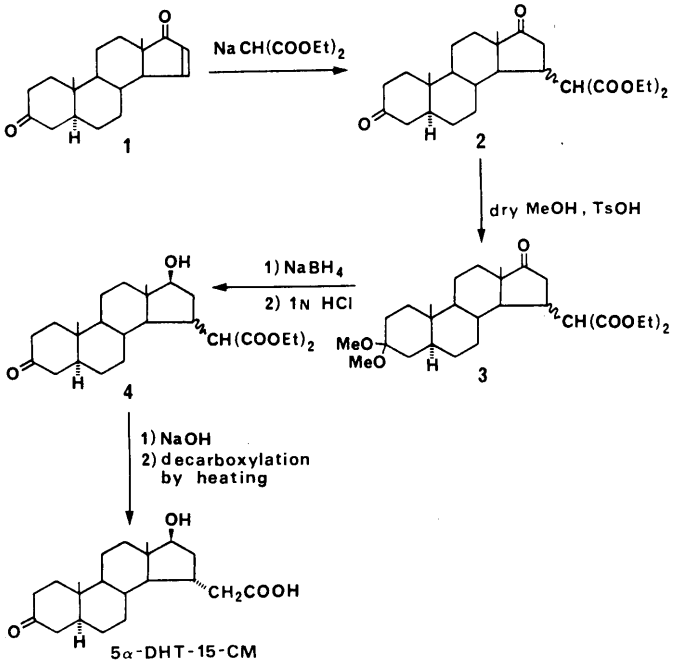

Chart 1. Preparation of 15α-Carboxymethyl-5α-dihydrotestosterone (5α-DHT-15-CM)

15-Bis(ethoxycarbonyl)methyl-5α-androstane-3,17-dione (**2**), obtained by reaction of 5α-androst-15-en-3,17-dione (**1**) with 1.5 mol equivalent of sodium diethyl malonate, was protected with dimethyl ketal to give the 3,3′-dimethoxy derivative (**3**). Reduction of **3** with sodium borohydride gave the 17β-hydroxy derivative (**4**). Decarboxylation of **4** led to the desired 5α-DHT-15-CM.

The stereochemistry of the C-15 substituent in 5α-DHT-15-CM is assigned as α based on molecular rotation analysis. The $[M]_D$ value (+258°) of 5α-DHT-15-CM revealed a dextrorotatory shift compared with that (+96°) of 17β-hydroxy-5-androstan-3-one. It is known that in a comparison of a 15-substituted steroid with the corresponding unsubstituted steroid, the 15β-substituent shows a large levorotatory shift, while the 15α-substituent shows a dextrorotatory shift. These findings were utilized in determination of the configuration at the C-15 position of 17-hydroxy steroids,[7] and 17-oxo steroids.[8]

The starting compound (**1**) was prepared according to the procedure of Rao *et al.*[9] and recrystallized from MeOH. mp 145 °C. IR (Nujol): 1710, 1720 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (6H, s, 18-Me and 19-Me), 6.03 (1H, dd, J=4, 5 Hz, 15-H), 7.47 (1H, dd, J=2, 5 Hz, 16-H). *Anal.* Calcd for $C_{19}H_{26}O_2$: C, 79.68; H, 9.15. Found: C, 79.44; H, 9.01.

15-Bis(ethoxycarbonyl)methyl-5α-androstan-3,17-dione (2) Diethyl malonate (8.43 g, 52 mmol) was added dropwise to a suspension of NaH (1.26 g, 52 mmol) in tetrahydrofuran (THF) (40 ml). A solution of **1** (10.1 g, 35 mmol) in THF (80 ml) was added, and the reaction mixture was stirred for 3 h at room temperature, then neutralized with acetic acid, poured into ice-water (1000 ml) and extracted with ethyl acetate. The ethyl acetate layer was washed with water, dried over Na_2SO_4 and evaporated *in vacuo* to give an oil. The oil was chromatographed on silica gel and eluted with ethyl acetate to give **2** (7.9 g, 50%), which was recrystallized from diethyl ether–*n*-hexane as colorless columns. mp 105 °C. $[\alpha]_D$ +66.1° (c=0.97, $CHCl_3$). IR (Nujol): 1740 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, s, 18-Me), 1.04 (3H, s, 19-Me), 1.25 (6H, t, J=8 Hz, $-CH(COOCH_2\underline{C}H_3)_2$), 3.87 (1H, d, J=4 Hz, $-C\underline{H}(COOEt)_2$), 4.18 and 4.19 (4H, q, J=8 Hz, $-CH(COO\underline{C}H_2CH_3)_2$). *Anal.* Calcd for $C_{26}H_{38}O_6$: C, 69.93; H, 8.58. Found: C, 69.74; H, 8.53.

15-Bis(ethoxycarbonyl)methyl-3,3′-dimethoxy-5α-androstan-17-one (3) *p*-Toluenesulfonic acid (27.2 mg) was added to a solution (130 ml) of **2** (3 g, 6.7 mmol) in dry MeOH. The mixture was stirred for 20 h at room temperature, neutralized with methanolic NaOH and evaporated *in vacuo*. The residual solid was dissolved in ethyl acetate (500 ml), washed with water and dried over Na_2SO_4. The ethyl acetate was evaporated *in vacuo* to give crude **3** (3.23 g, 98%), which was used for the following step without further purification. IR (Nujol): 1740 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-Me), 0.94 (3H, s, 19-Me), 1.25 (6H, t, J=8 Hz, $-CH(COOCH_2\underline{C}H_3)_2$), 3.12 and 3.17 (6H, s, $-C(O\underline{C}H_3)_2$) 3.89 (1H, d, J=4 Hz, $-C\underline{H}(COOEt)_2$), 4.16 and 4.20 (4H, q, J=8 Hz, $-CH(COO\underline{C}H_2CH_3)_2$).

15-Bis(ethoxycarbonyl)methyl-17β-hydroxy-5α-androstan-3-one (4) A solution of **3** (3.2 g) in dry EtOH (100 ml) was added dropwise to a stirred solution of sodium borohydride (0.55 g) in dry EtOH (65 ml) and the mixture was stirred for 7 h at 0 °C. Excess sodium borohydride was decomposed by addition of 1 N HCl. The solution was poured into ice-water and extracted with ethyl acetate. The organic solution was washed with water, dried over Na_2SO_4 and concentrated to about 40 ml *in vacuo*. To the concentrate was added 1 N HCl (40 ml), and the mixture was stirred for 2 h at room temperature. The ethyl acetate layer was washed with water, dried over Na_2SO_4 and evaporated *in vacuo* to give **4** (1.71 g, 63%), which was recrystallized from diethyl ether–*n*-hexane as colorless needles. mp 113 °C. $[\alpha]_D$ +66.1° (c=1.05, EtOH). IR (Nujol): 3440 (OH), 1720 and 1740 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.82 (3H, s, 18-Me), 1.04 (3H, s, 19-Me), 1.25 and 1.27 (6H, t, J=8 Hz, $-CH(COOCH_2\underline{C}H_3)_2$), 1.74 (1H, s, –OH), 3.67 (1H, t, J=8 Hz, 17α-H), 3.68 (1H, d, J=4 Hz, $-C\underline{H}(COOEt)_2$), 4.14 and 4.21 (4H, q, J=6 Hz, $-CH(COO\underline{C}H_2CH_3)_2$). *Anal.* Calcd for $C_{26}H_{40}O_6$: C, 69.61; H, 8.99. Found: C, 69.26; H, 8.95.

15α-Carboxymethyl-17β-hydroxy-5α-androstan-3-one (5α-DHT-15-CM) A solution of **4** (617 mg) in distilled EtOH was treated with 1 N NaOH (2.0 ml). The mixture was stirred for 4 h at room temperature, neutralized with 1 N HCl and extracted with ethyl acetate. The ethyl acetate layer was washed with water and dried over Na_2SO_4. After removal of the solvent, the residual solid was dissolved in nitrobenzene (30 ml) and heated for 1.5 h at 145 °C. Nitrogen gas was bubbled through the solution during the heating. After removal of the solvent *in vacuo*, the residual oil was chromatographed on silica gel and eluted with $CHCl_3$–EtOH (98:2) to give an oil. This oil was dissolved in dioxane (8 ml), 1 N NaOH (2.63 ml) was added, and the mixture was stirred for 6 h at room temperature. The mixture was adjusted to pH 3 with 1 N HCl and extracted with ethyl acetate. Th ethyl acetate layer was washed with water and dried over Na_2SO_4. After removal of the solvent, the residue was chromatographed on silica gel and eluted with $CHCl_3$–EtOH (95:5) to give 5α-DHT-15-CM (256 mg, 55%) which was recrystallized from MeOH as columns. mp 231 °C. $[\alpha]_D$ +74.2° (c=0.6, EtOH). IR (Nujol): 3360 (OH), 1710 and 1720 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-Me), 1.08 (3H, s, 19-Me), 3.56 (1H, t, J=8 Hz, 17α-H). *Anal.* Calcd for $C_{21}H_{32}O_4$: C, 72.38; H, 9.26. Found: C, 72.27; H, 9.26.

Preparation of D-GL Conjugates Preparation of 5α-DHT-15-CM and 15α-CMT coupled to D-GL copolymers was done according to the mixed anhydride method described by Erlanger *et al.*[1]

Immunization Procedures and Collection of Antisera Six groups of female New Zealand white rabbits, with 3 animals per group, received a primary immunization subcutaneously at multiple sites along the back with 1 mg of the 15α-CMT–BSA conjugate (groups 1, 2 and 3) or 15β-CMT–BSA conjugate (groups 4, 5 and 6) emulsified with complete Freund's adjuvant. Groups 1—3 and 4—6 were boosted with 0.5 mg of 15α- and 15β-CMT–BSA, respectively, emulsified with complete Freund's adjuvant every 4 weeks. Groups 2 and 3 were pretreated with single intraperitoneal injections (i.p.) of 5 mg of 5α-DHT-15-CM–D-GL and 15α-CMT–D-GL, respectively, in saline 3 d before the primary immunization with 15α-CMT–BSA. Groups 5 and 6 were pretreated with single i.p. injections of 5α-DHT-15-CM–D-GL and 15α-CMT–D-GL, respectively, in saline 3 d before the primary immunization with 15β-CMT–BSA. Groups 1 and 4 received saline without the D-GL conjugate 3 d before primary immunization. Blood was collected 3 months after the primary immunization and centrifuged at 2500 rpm for 10 m.

Assay Procedure All dilutions of the standard, labeled antigen and antiserum were prepared with 0.1 M borate buffer (pH 8.0) containing 0.1% gelatin, 0.9% NaCl and 0.01% NaN_3. [^{3}H]Testosterone (20000 dpm, *ca.* 45 pg) (0.1 ml) and diluted antiserum (0.1 ml) were added to a series of standard solutions (0.2 ml) and the mixtures were incubated for 2 h at room temperature. After addition of dextran-coated charcoal containing gelatin (0.5 ml) (dextran 0.05%, charcoal 0.5%, gelatin 0.1%), the suspension was vortex-mixed, allowed to stand at 4 °C for 10 m, and then centrifuged for 30 m at 2500 rpm. The radioactivity in the supernatant (0.5 ml) was measured with a Aloka ES-700 liquid scintillation spectrometer.

Results and Discussion

A titer of each antiserum obtained was tested at various dilutions to determine the 50% binding level. The rabbits (groups 1 and 4) treated with saline, which was free from 5α-DHT-15-CM–D-GL, and the rabbits (groups 2 and 5) treated with 5α-DHT-15-CM–D-GL 3 d before primary immunization had detectable titers of anti-testosterone antisera, but the rabbits (groups 3 and 6) treated with 15α-CMT–D-GL 3 d before primary immunization did not. The

Table I. Titers of Anti-15α- and 15β-CMT Antisera

Antiserum	Group	Treatment	Rabit No.	Titer
Anti-15α-CMT antiserum	1	Saline	13	1: 9800
			14	1:12000
			15	1:22000
	2	5α-DHT-15-CM–D-GL	16	1: 6400
			17	1: 4100
			18	1: 7200
	3	15α-CMT–D-GL	19—21	Not detectable
Anti-15β-CMT antiserum	4	Saline	22	1:21000
			23	1:14000
			24	1:10000
	5	5α-DHT-15-CM–D-GL	25	1: 3700
			26	1: 6400
			27	1: 6400
	6	15α-CMT–D-GL	28—30	Not detectable

Titers are expressed as the reciprocal of the dilution of antisera binding 50% of added [^{3}H]testosterone (*ca.* 20000 dpm). Abbreviations for steroids: 15α- and 15β-CMT are 15α- and 15β-carboxymethyltestosterone, respectively. 5α-DHT-15-CM–D-GL is 15β-carboxymethyl-5α-dihydrotestosterone linked to a copolymer of D-glutamic acid and D-lysine.

TABLE II. Percentage Cross-Reactivity of Anti-15α-CMT Antisera

Steroid	Group 1			Group 2		
	No. 13	No. 14	No. 15	No. 16	No. 17	No. 18
Testosterone	100.00	100.00	100.00	100.00	100.00	100.00
5α-Dihydrotestosterone	4.26	3.98	7.65	0.73	1.01	1.37
5β-Dihydrotestosterone	0.40	0.23	0.35	0.27	0.43	0.22
4-Androstene-3β,17β-diol	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
5α-Androstane-3β,17β-diol	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
4-Androstene-3,17-dione	0.69	0.64	0.78	0.77	0.77	0.52
11β-Hydroxytestosterone	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Progesterone	0.44	0.40	0.52	0.45	0.34	0.40
Deoxycorticosterone	0.74	0.43	0.47	0.59	0.71	0.30
Cortisol	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Corticosterone	0.03	0.02	0.02	0.03	0.03	0.01
Estrone	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Estradiol	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

TABLE III. Percentage Cross-Reactivity of Anti-15β-CMT Antisera

Steroid	Group 4			Group 5		
	No. 22	No. 23	No. 24	No. 25	No. 26	No. 27
Testosterone	100.00	100.00	100.00	100.00	100.00	100.00
5α-Dihydrotestosterone	6.60	6.91	3.05	0.57	1.27	0.83
5β-Dihydrotestosterone	0.55	0.31	0.46	0.52	0.33	0.21
4-Androstene-3β,17β-diol	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
5α-Androstane-3β,17β-diol	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
4-Androstene-3,17-dione	0.77	0.82	0.68	0.80	0.68	0.68
11β-Hydroxytestosterone	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Progesterone	0.48	0.21	0.45	0.37	0.49	0.51
Deoxycorticosterone	0.40	0.73	0.20	0.59	0.53	0.20
Cortisol	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Corticosterone	0.06	0.03	0.02	0.02	0.02	0.04
Estrone	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Estradiol	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

titers are shown in Table I.

The specificity of the resulting antisera was evaluated by ascertaining the ability of various related steroid to compete with [^{3}H]testosterone for binding to the antibody. The cross-reaction of antiserum was determined using the method of Abraham[10] and the data are shown in Tables II and III.

The anti-15α-antiserum produced without pretreatment (group 1) showed a range of 4.26—7.65% (mean 5.30± 1.67%) cross-reaction with 5α-DHT, and other steroids that showed low cross-reaction included progesterone (0.40—0.52%), deoxycorticosterone (0.43—0.74%), corticosterone (0.02—0.03%) and androstenedione (0.64—0.78%). On the other hand, the anti-15α-CMT antiserum produced by pretreatment with 5α-DHT-15-CM-D-GL (group 2) showed a range of 0.73—1.37% (mean 1.04±0.26%) cross-reaction with 5α-DHT, and other steroids that showed low cross-reaction included progesteron (0.34—0.45%), deoxycorticosterone (0.30—0.71%), corticosterone (0.01—0.03%) and androstenedione (0.52—0.77%). The anti-15β-antiserum produced without pretreatment (group 4) showed a range of 3.05—6.91% (mean 5.52±1.75%) cross-reaction with 5α-DHT, and other steroids that showed low cross-reaction included progesterone (0.21—0.48%), deoxycorticosterone (0.20—0.73%), corticosterone (0.02—0.06%) and androstenedione (0.68—0.82%).

On the other hand, the anti-15β-CMT antiserum produced by pretreatment with 5α-DHT-15-CM-D-GL (group 5) showed a range of 0.57—1.27% (mean 0.89±0.29%) cross-reaction with 5α-DHT, and other steroids that showed low cross-reaction included progesterone (0.37—0.51%), deoxycorticosterone (0.20—0.59%), corticosterone (0.02—0.04%) and androstenedione (0.68—0.80%).

The cross-reactivity with 5α-DHT (mean 1.04±0.26%) of anti-15α-CMT antiserum produced by pretreatment with 5α-DHT-15-CM-D-GL was lower than that (mean 5.30± 1.67%) of anti-15α-CMT antiserum produced without pretreatment. The cross-reactivity with 5α-DHT (mean 0.89±0.29%) of anti-15β-CMT antiserum produced by pretreatment with 5α-DHT-15-CM-D-GL was also lower than that (mean 5.52±1.75%) of anti-15β-CMT antiserum produced without pretreatment. Accordingly, pretreatment with 5α-DHT-15-CM-D-GL conjugate was quite effective in suppressing selectively the production of antibody to 5α-DHT. On the other hand, each anti-15α-CMT and anti-15β-CMT antiserum prepared by the pretreatment method showed high specificity for C-21 steroids, and the specificities were similar to those of the respective antisera obtained without pretreatment.

References

1) B. F. Erlanger, F. Borek, S. M. Beiser and S. Lieberman, *J. Biol. Chem.*, **228**, 713 (1957).
2) S. G. Hillier, B. G. Brownsey and E. H. D. Cameron, *Steroids*, **21**, 735 (1973); J. J. Pratt, T. Wiegman, R. E. Lappohn and M. G. Woldring, *Clin. Chim. Acta*, **59**, 337 (1975); P. N. Rao, P. H. Moore, Jr., D. M. Peterson and R. K. Tcholakian, *J. Steroid Biochem.*, **9**, 539 (1978).
3) Y. Miyake, Y. Kubo, S. Iwabuchi and M. Kojima, *Steroids*, **40**, 245 (1982).
4) K. Tateishi, T. Hamaoka, C. Hayashi, J. Goto, S. Ikegawa and T. Nambara, *Steroids*, **36**, 238 (1980).
5) D. H. Katz, T. Hamaoka and B. Benacerraf, *J. Exp. Med.*, **136**, 1404 (1972); T. Hamaoka and D. H. Katz, *ibid.*, **139**, 1446 (1974).
6) J. Dupret, C. Grenot and M. Rolland, *J. Steroid Biochem.*, **20**, 1345 (1984).
7) M. Matsui and Y. Kinuyama, *J. Chem. Soc., Perkin Trans. 1*, **1976**, 1429.
8) E. W. Cantrall, R. Littell and S. Benstein, *J. Org. Chem.*, **29**, 64 (1964); P. N. Rao and P. H. Moore, Jr., *Steroids*, **28**, 101 (1976).
9) P. N. Rao, A. H. Khan and P. H. Moore, Jr., *Steroids*, **29**, 171 (1977).
10) G. E. Abraham, *J. Clin. Endocrinol.*, **29**, 866 (1969).