A RADIOIMMUNOASSAY FOR PLASMA TESTOSTERONE

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ABSTRACT

A simple and reliable radioimmunoassay for plasma testosterone has been developed. Antiserum against testosterone was produced in rabbits immunized with testosterone-3-oxime-beef serum albumin. Plasma volumes of 0.1 ml from men and 0.5 ml from women were used for analysis. After a preliminary solvent wash, the plasma was extracted and the extracts were applied to Al_2O_3 micro columns. Following elution, the dried purified extract was incubated with antiserum at room temperature for 2 hours. $(NH_4)_2SO_4$ was used to separate free from bound testosterone. 1,2-^{3}H-testosterone was used for correction of losses and for determination of the % bound. The accuracy and precision of the method were satisfactory. The sensitivity was 10 pg per sample. The mean blank was 2 ± 2 (SD) pg per sample. Analysis of plasma duplicates by an alternative method utilizing paper chromatography for purification of extracts did not give results that differed significantly from those obtained with the present method. The mean and range of plasma testosterone concentrations in normal men and women determined by this method were similar to those reported by other investigators using different methods.

INTRODUCTION

In recent years, numerous techniques have been developed for measuring plasma testosterone with double isotope derivative methods (1), gas-liquid chromatography (2), and competitive protein binding using testosterone binding globulin (3). Some of these methods are accurate, precise, sensitive and specific; however, many are extremely tedious and all require at least one thin layer, paper, or gas-liquid chromatography for purification of the plasma extract in order to measure testosterone in plasma from men and women.

This report describes a reliable radioimmunoassay for testosterone in plasma from men and women which uses for purification only solvent partitioning and adsorption chromatography on a micro column of Al_2O_3.

Steroid-protein conjugates have been used as antigens (4) to develop antibodies which, in some cases, are more specific for certain steroids than are naturally occurring plasma binding proteins (5). Recently, radioimmunoassay methods have been reported for plasma estradiol (6), estrone (7), and aldosterone (8). The radioimmunoassay of testosterone was investigated by Niswender and Midgeley but details have not been published (9).

MATERIALS

The source and purification of 1,2-^{3}H-testosterone (51.1 c/m mole) (10) and the composition of the scintillation fluid (11) were previously described. Testosterone was obtained from Sigma Chemical Co. Methanol (J. T. Baker Chemical Co.), 95% ethanol (Commercial Solvent Co.), dichloromethane (Matheson, Coleman and Bell) and hexane (Mallinckrodt) were redistilled once. Absolute ethanol was obtained from Commercial Solvent Co. and used without purification. Ether (Mallinckrodt) was redistilled over sodium bisulfite, stored in a brown bottle at 4° C, and used within 2 weeks. Other materials and their sources are carboxymethoxylamine hemi-HCl (K and K Laboratories), bovine serum albumin (BSA, Mann Research Laboratories), complete Freund's adjuvant (Difco) and human gamma globulin (Parke, Davis, and Co.). The Al_2O_3 for chromatography (Savory and Moore, Brockmann grade 2-3, mesh 100-150) was purified as described earlier (10).

METHODS

Washing of glassware

New glassware, or glassware that had come in contact with plasma samples or antisera, was washed with soap and water, soaked in dilute HCl overnight, rinsed with distilled water, and dried in an oven at 60° C. Pipettes used for dispensing solvents or for measuring purified plasma extracts after column chromatography were washed with methanol: dichloromethane (1:1) just after use and stored in a covered cylinder of the same solvent mexture. Tubes used for collecting eluates were rinsed with ethanol just after use and stored dry. Just before use, all tubes and pipettes were washed with methanol:dichloromethane (1:1).

1,2-^{3}H-testosterone

Purified 1,2-^{3}H-testosterone was diluted with ethanol so that 0.1 ml contained 35,000 DPM. After dispensing this solution in tubes, the ethanol was dried in a vacuum oven at 40° C and 5 mm of mercury pressure before the tubes were used.

Preparation of Al_2O_3

Glass columns, 11 cm long and 0.5 cm in internal diameter, were prepared from disposable glass pipettes by shortening the constricted portions. Each tube was packed with 3 cm of Al_2O_3 after plugging the constricted portion with glass wool. This glass wool was packed tight-

ly so that at the time of elution of testosterone from the column, the flow rate was 1.6 ml in 5 minutes. Columns were washed twice with ethanol, 4 times with methanol, 3 times with methanol:dichloromethane (1:1) and 3 times with dichloromethane just before use. Each wash volume was 1.6 ml. Columns were used repeatedly for a period of more than 6 months.

Antiserum

Testosterone-3-oxime-bovine serum albumin was prepared by the method of Erlanger et al (12). One mg of conjugate in 0.5 ml of isotonic saline was emulsified with 0.5 ml of complete Freund's adjuvant. This amount was injected into each rabbit in 4 intramuscularly sites once a week for 3 weeks, twice a month for one month, and subsequently once a month. Of the 6 animals immunized, 2 developed antisera that could be used for radioimmunoassay at a dilution of greater than 1/5,000. The antiserum used for this report was obtained from one rabbit 10 days after the 7th injection.

The antiserum was preabsorbed with BSA before use. Five mg of BSA were added to 1 ml of antiserum diluted 1:10 with 0.05 M borate buffer, pH 8, and incubated for 24 hours at 4° C. After centrifugation at 2,000 g for 15 minutes, the supernatant was removed and stored at -10° C. Just before use the BSA-treated antiserum was diluted 1:750 with 0.05 M borate buffer, pH 8, containing 0.06% BSA and 0.05% human gamma-globulin. Omission of BSA from the buffer resulted in adsorption of unbound 1,2-^{3}H-testosterone onto either walls of glass tubes or protein precipitates after addition of $(NH_4)_2SO_4$, as had been observed earlier in the assay of estradiol (11). Gamma globulin was added to increase the mass of the precipitate.

Extraction and purification

One tenth milliliter of plasma from men was added to 3 ml conical glass tubes containing 35,000 DPM of 1,2-^{3}H-testosterone. One half ml of plasma from women was used in 15 ml conical glass tubes containing 17,500 DPM of 1,2-^{3}H-testosterone. Smaller tubes were used for samples from men in order to facilitate separation of solvent from the small aliquots of plasma. The samples were pre-extracted by shaking with 4 volumes of hexane for 10 minutes on a mechanical shaker. After sitting for 1 minute the organic phase was aspirated and discarded. The washed plasma was extracted with 10 volumes of hexane:ether (8:2) by shaking on a mechanical shaker for 10 minutes. After sitting for 1 minute the organic phase was transferred directly to Al_2O_3 micro columns with a pipette. Columns were washed once with 1.6 ml of hexane:ether (8:2) and 4 times with 1.6 ml portions of 0.45% absolute ethanol in hexane. Testosterone was eluted from the Al_2O_3 with two 1.6 ml portions of 0.95% absolute ethanol in hexane. Volumes for washing and elution on the micro column were measured by using the estimate that the space in disposable pipettes above the Al_2O_3 was 1.6 ml. The micro columns were allowed to run dry each time they were filled with solvent.

Radioimmunoassay

Eluates from the columns were mixed thoroughly on a 'Vortex' mixer and duplicate aliquots containing approximately 3,500 DPM of 1,2-^{3}H-testosterone were transferred to 2 ml fine point conical tubes. For determination of the total radioactivity transferred to eluate assay tubes, the same aliquot was added to a counting vial and evaporated to dryness. Eluate volumes of 0.5 ml for samples from men and 1 ml for samples from women were usually satisfactory. One tenth milliliter

volumes of standard solutions in ethanol containing 0, 25, 50, 80, 100 and 150 pg of testosterone and 3,500 DPM of 1,2-^{3}H-testosterone were added to another set of 2 ml fine point conical tubes. For determination of the total radioactivity added to standard assay tubes, the volume of a standard solution containing nominally 3,500 DPM of 1,2-^{3}H-testosterone was added to a counting vial and evaporated to dryness.

The assay tubes containing aliquots of eluates and standards were dried in a vacuum oven at 40° C and 5 mm of Hg. For assay, 0.25 ml of diluted antiserum (1:7,500) was added to each assay tube and the contents were mixed thoroughly on a 'Vortex' mixer. The tubes were covered with 'Parafilm' (American Can Co.) and were allowed to sit at room temperature for 2 hours. To separate free from bound testosterone, 0.25 ml of saturated $(NH_4)_2SO_4$ was added to each tube and the tubes were immediately mixed on a 'Vortex'. Foaming was avoided. After the tubes were again covered with 'Parafilm' and allowed to sit for 10 minutes at room temperature, they were centrifuged for 10 minutes at 2,000 g. The supernatants were transferred with disposable pipettes, without disturbing the precipitates, to 2 ml plastic 'Technicon' disposable cups. One quarter milliliter of each supernatant was then transferred to a counting vial. One quarter milliliter of half saturated $(NH_4)_2SO_4$ was added to all counting vials containing dried eluates or dried standards for determination of total recovery. After addition of 10 ml portions of scintillation fluid, the vials were shaken for 15 minutes and counted to 20,000 counts.

Calculations

The standard curve was constructed by plotting the percent of bound steroid as a function of the mass of unlabelled testosterone (Figure 1). The percent of bound steroid of standards equals (1-2F/S) x 100, and of samples equals (1-2F/A). In these equations F (indicating free) is the DPM in the 0.25 ml aliquot of supernatant after precipitation of bound testosterone with $(NH_4)_2SO_4$, S is the total DPM in the standards, and A is the total DPM in the aliquot of eluate used for analysis. The uncorrected testosterone content of samples was read off the standard curve. Corrections were made for losses in the method and for the difference between the mass of 1,2-^{3}H-testosterone in the standards and that in the eluates of sample extracts used for analysis. The 1,2-^{3}H-testosterone used had a specific activity of 390 DPM/pg.

$$\text{pg in plasma sample} = \left(\text{the amount read off the standard curve} - \frac{A - S}{390}\right) \times \frac{T}{A}$$

In this equation, T is the total DPM added to the original plasma sample prior to extraction.

RESULTS

Recovery

The mean recovery of 1,2-^{3}H-testosterone added to 49 plasma samples was 54.6 $\pm$ 5.9 (SD)%.

Standard Curve

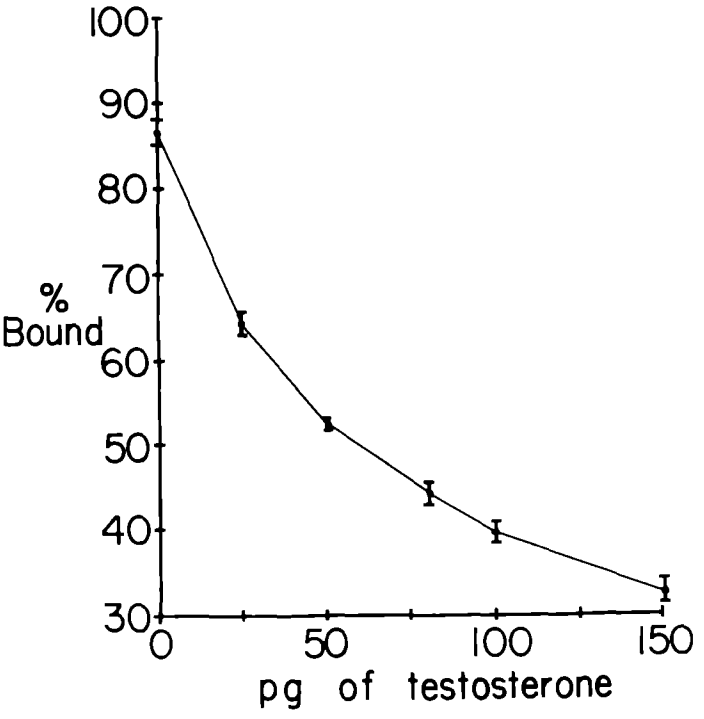

Figure 1. Standard Curve. The percent of bound 1,2-^{3}H-testosterone is plotted as a function of the amount of unlabelled testosterone in each tube. The points represent the means of 5 replicates done in the same run. The bars indicate 2 SD on either side of the mean.

To examine the reproducibility of standards within a run, 5 standards were assayed at each concentration. The results are shown in Figure 1. The variability of the standard curve from day to day was small. In the last 5 successive standard curves done on separate days, the means $\pm$ SD of the % of bound steroid for the 0, 80, and 150 pg standards were 85.5 $\pm$ 1.6%, 43.1 $\pm$ 2.0% and 31.6 $\pm$ 2.7%.

Accuracy

A pool of ether-extracted plasma (11) was used to measure accuracy. Zero, 100, 300 and 500 pg of testosterone were added to five 0.5 ml aliquots of the pool. The relation of testosterone added to testosterone measured is shown in Table I. The analysis of variance gave the

following equation:

pg of testosterone measured = 2 + 0.985 times pg of testosterone expected

TABLE I.

Accuracy: the results of determination of testosterone added to ether extracted plasma (pg per sample)

Testosterone added	Testosterone found Individual values					Mean ± S.D.
0	1	3	3	0	1	1.6 ± 1.8
100	91	91	105	101	98	97 ± 6
300	280	307	337	281	318	304 ± 24
500	497	485	522	475	477	491 ± 19

Precision

Intra-assay precision was measured by examining the results of the accuracy study (Table I). The coefficients of variation for analysis of 500, 300 and 100 pg amounts added to ether-extracted plasma were 3.9, 7.9 and 6.2% respectively and the SD of the blank was 2 pg. Intra assay precision was also examined by measuring the testosterone concentrations of replicates from pools of plasma from men and women. The mean ± SD of testosterone concentration of 7 samples from a pool of plasma from men and 7 from a pool from women were 794 ± 31 ng/100 ml (coefficient of variation 3.9%) and 30.3 ± 2.8 (SD) ng/100 ml (coefficient of variation 9.2%), respectively. Inter-assay precision was examined by analysis of the testosterone concentration in duplicate plasma specimens run on separate days. Eight of these specimens were obtained from men and six from women. The mean difference observed was 7.4 ± 6.1 (SD)%.

Sensitivity

Ten pg of testosterone were added to four 0.5 ml aliquots of a pool of ether-extracted plasma which initially contained a mean of 3 pg of testosterone per 0.5 ml. The mean testosterone found in the reinforced plasma samples was 10 $\pm$ 1 (SD) pg. The difference between the unreinforced and reinforced plasma means was 7 pg ($p < 0.02$).

Specificity

The specificity of the method was examined by analysis of blanks, by study of antibody specificity, and by comparison of results obtained with the present method with those obtained using established methods. The mean $\pm$ SD of testosterone found in ten 0.5 ml volumes of ether-extracted distilled water examined on separate days was 2 $\pm$ 2 pg. The mean $\pm$ SD of testosterone in five 0.5 ml aliquots of ether-extracted plasma examined in the same run was 2 $\pm$ 2 pg. Thirty-two steroids were examined for competition with testosterone for displacement of 1,2-^{3}H-testosterone from the antibody. The eight steroids which caused the most marked interference are shown in Figure 2. The antibody had considerable specificity for testosterone. However, at the level of 50% bound, dihydrotestosterone (17β-hydroxy-5α-androst-3-one, DHT in Figure 2), 17β-hydroxy-androst-1,4-dien-3-one (delta-1-testosterone, a in Figure 2) and 17β-hydroxy-5α-androst-1-en-3-one (delta-1-DHT, b in Figure 2) were equivalent to 42, 38 and 21%, respectively, of their weight of testosterone on direct incubation with the antibody. To further investigate interference quantitatively, 1,000 pg of these same 3 steroids were added in duplicate to 0.5 ml volumes of ether-extracted plasma. One nanogram of DHT processed through the method was measured as though it were equivalent to 231 pg of testosterone. Similar amounts of delta-1-testosterone and of delta-1-DHT were measured as though they

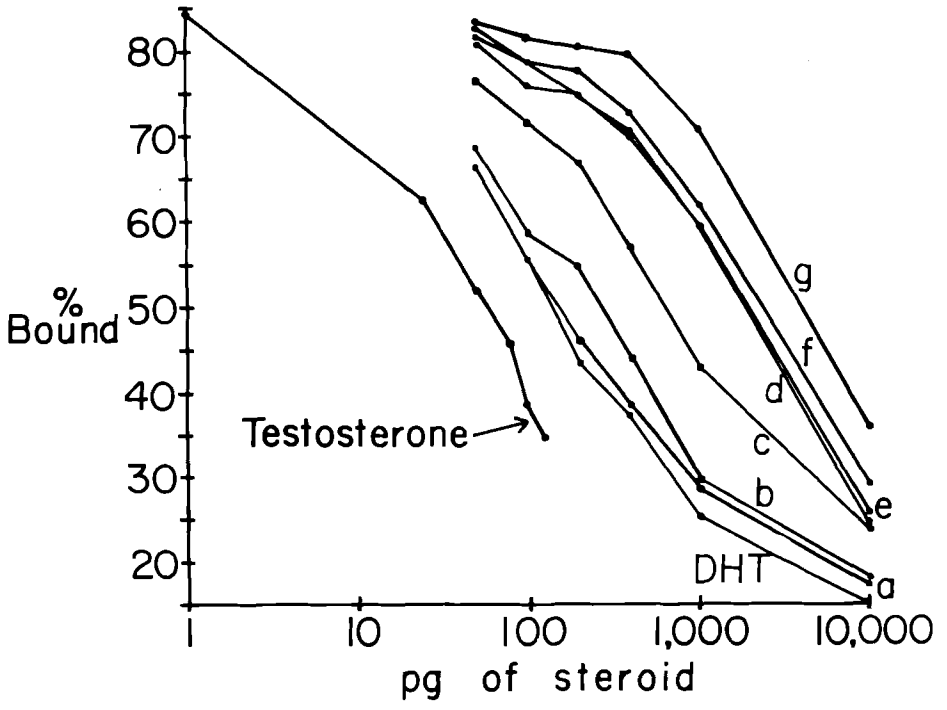

Figure 2. Standard curves for testosterone and the 8 steroids with the most marked interference. The percent of bound 1,2-^{3}H-testosterone is plotted as a function of the amount of unlabelled steroid in each tube.
a. 17β-hydroxy-androst-1,4-dien-3-one
b. 17β-hydroxy-5α-androst-1-en-3-one
c. 17β-hydroxy-5β-androstan-3-one
d. 4-androsten-3β,17β-diol
e. 5α-androstan-3α,17β-diol
f. 4-androsten-3,17-dione
g. 5-androsten-3β,17β-diol.

were equivalent to 50 and 242 pg of testosterone, respectively. Five other C_{19} steroids were identified (c. through g. in Figure 2) which were equivalent, on direct incubation with antibody, to between 1.5 and 9% of their weight of testosterone at a level of 50% bound. In addition twenty-four C_{18}, C_{19} and C_{21} steroids were shown to have little affinity for the antibody (Table II). When these 24 steroids, in 10 ng amounts, were added to the final assay tube for incubation with the antibody, none was equivalent to as much as 50 pg of testosterone. In Table III,

TABLE II

Steroids with Less Marked Interference*

C_{18} steroids: estrone, estradiol, estriol.
C_{19} steroids: 3β-hydroxy-5α-androstan-17-one, 5β-androstan-3α, 17β-diol, 3α-hydroxy-5β-androstan-17-one, 3α-hydroxy-5β-androstan-11,17-dione, 3α,11β-dihydroxy-5β-androstan-17-one, 5α-androst-1-en-3,17-dione, 4-androsten,3,11,17-trione, 17α-hydroxy-androst-4-en-3-one, 3β-hydroxy-androst-5-en-17-one, androsterone, dehydroepiandrosterone.
C_{21} steroids: progesterone, 17α-hydroxy-progesterone, 5β-pregnan-3α,20α-diol, 5β-pregnan-3α,17α ,20α-tiol, 3α,17α,20α-trihydroxy-5β-pregnan-11,22-dione, 19-nor-progestrone, 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one, corticosterone, deoxycorticosterone, cortisol, cortisone, 11-deoxycortisol, aldosterone.

* Ten ng of these steroids, when incubated directly with antiserum and 1,2-^{3}H-testosterone, were equivalent to less than 50 pg of testosterone.

TABLE III. Specificity: Testosterone Concentrations Determined Using Different Methods*
ng/100 ml of plasma

	Plasma from Men		Plasma from Women	
	Column + Antibody	Paper + TBP	Column + Antibody	Paper + TBP
	794	735	35.0	30.2
	649	648	36.8	32.8
	731	718	38.2	38.5
	611	545	21.5	26.0
	401	406	30.3	27.4
	423	449	31.9	39.8
	365	392	40.2	46.3
	450	470		
Mean	553	545	33.3	34.4

* Column + antibody refers to the method described in this report. Paper + TBP refers to the method using testosterone binding protein described earlier (10) with purification by paper chromatography but omitting thin layer chromatography.

the plasma testosterone concentrations determined by the present method (Column + antibody) are compared with the results of analyses of duplicate specimens determined by a modification of the method of Mayes and Nugent (10) using testosterone binding protein (Paper + TBP). In the original method (10), plasma extracts were purified by paper and thin layer chromatography before incubation with testosterone binding protein.

For the present study, the thin layer chromatography was omitted since it has been shown that this omission did not result in a significant error (10). The means of the results (Table III) obtained with the present method were not significantly different from those obtained with the modified method of Mayes and Nugent in the analysis of plasma from men and women ($p > 0.5$). In Table IV the testosterone concentrations in the plasma of normal men and women determined by the present method are compared with the results reported by others using different methods (1, 10, 13, 14).

DISCUSSION

The reproducibility of the standard curve in the same run and on separate days is close. A slightly steeper standard curve can be obtained by performing the testosterone-antibody incubation at 4° C for 16 hours and by fractionating with $(NH_4)_2SO_4$ at the same temperature. However, the standard curve in Figure 1 is adequate for measurement of testosterone in 0.5 ml aliquots of plasma from women.

The accuracy of the method was examined throughout the concentration range from 0 to 500 pg per sample. No major systematic error in the method was found by the analysis of variance and the intercept of the regression line with the Y axis is close to zero.

The intra-assay and inter-assay precision of the method are comparable to those reported for reliable competitive protein binding methods for plasma testosterone using testosterone binding protein (3).

The sensitivity of the method is adequate for measuring testosterone in 0.1 and 0.5 ml plasma volumes from men and women, respectively.

The specificity of the method, examined 3 different ways, seems adequate. Both ether-extracted water and ether-extracted plasma blanks

TABLE IV. Plasma testosterone concentrations in ng/100 ml in normal subjects*

Author	Method	Female				Male				Water blank
		Mean	± SD	Range	No.	Mean	± SD	Range	No.	
Kirschner et al. (13)	Double isotope dilution using acetic anhydride	70	30	20-120	10	740	260	440-1,300	15	10
Rivarola and Migeon (1)	Double isotope dilution using acetic anhydride	47	15	25-68	9	551	151	373-917	13	7
Kobayashi et al. (14)	Double isotope dilution using thiosemicarbazide	37	17		77					16
Mayes and Nugent (10)	Competitive protein binding using testosterone binding protein	40	14	20-76	18	680	180	450-960	16	0.4
Present authors	Radioimmunoassay	32	7	22-45	12	590	149	365-815	13	0.4

* The blank has been subtracted from the values of all except the lower two reports.

are very low. In this method, blanks are not subtracted from plasma values to calculate results. The specificity of the antibody is good but 3 steroids were found which cause substantial interference with the binding of testosterone to the antibody. Of the 3 steroids, DHT has been found in plasma (15) while the natural occurrence of delta-1-testosterone and of delta-1-DHT would be unexpected. Due to a lesser affinity of DHT for the antibody and a greater loss of DHT than of testosterone in the purification process, 1 ng of DHT in plasma samples processed through the method is measured as though it is equivalent to 23.1% of its weight of testosterone. The mean concentration of DHT in plasma from men and women is 54 and 12 ng/100 ml, respectively (15). Therefore, the mean error attributable to the non-specific measurement of DHT by this method amounts to a positive error in determined testosterone concentration of only 12.4 and 2.8 ng/100 ml in plasma from men and women respectively.

The results of assay of plasma specimens by the present method were not significantly different from those found on analysis of duplicate specimens by a modification of the method of Mayes and Nugent (10) utilizing paper chromatography for purification of extracts. Also, the concentrations of plasma testosterone determined using this method in samples from normal men and women were similar to the values reported by other investigators using different methods.

Changes in the structure of testosterone have variable effects on the binding affinity of the steroid for the antibody. The following alterations are listed in order of increasing effectiveness in decreasing binding with the antibody: reduction of the 4,5-bond with formation of 5α-androstane, desaturation of the 1,2-bond, desaturation of the

1,2-bond and reduction of the 4,5-bond with formation of 5α-androstane, reduction of the 4,5-bond with formation of 5β-androstane, 3-keto to 3β-hydroxy, reduction of 4,5-bond with formation of 5α-androstane and 3-keto to 3α-hydroxy, 17β-hydroxy to 17-keto, shift of the double bond from 4,5 to 5,6 and 3-keto to 3β-hydroxy, and 17β-hydroxy to 17α-hydroxy. Midgley and Niswender (5) have made the generalization that alterations in steroid molecules far from the site of conjugation have greater influence on binding affinity than alterations near the site of conjugation. Our findings are consistent with this generalization.

In setting up this method in another laboratory, particularly if it is not possible to use Al_2O_3 from the specified source, it might be necessary to readjust the solvents used for washing and eluting the Al_2O_3 column. The concentration of ethanol in the last wash before elution should be adjusted so that approximately 15% of the testosterone in the extract applied to the Al_2O_3 column would be removed from the column and discarded in the washings. The concentration of ethanol in the eluting solvent should be adjusted so that approximately 15% of the testosterone added to the column remains on the column after elution. Not all sources of Al_2O_3 can be expected to give satisfactory purification. Initially, duplicate samples should be analyzed by both the present method and by some other method incorporating either a thin layer or a paper chromatography to verify that the Al_2O_3 columns function properly.

One technician can complete analysis of 20 plasma samples plus 2 samples of ether-extracted plasma fortified with known amounts of testosterone in addition to a set of direct standards in 1½ working days.

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REFERENCES

1. Rivarola, M. A., and Migeon, C. J., STEROIDS 7, 103 (1966).
2. Wotiz, H. H., and Clark, S. J., METHODS OF BIOCHEMICAL ANALYSIS 18, 357 (1970).
3. Nugent, C. A., and Mayes,D., ACTA ENDOCRINOL. Supp. 147, 257,(1970).
4. Liberman, S., Erlanger, B. F., Bieser, S. M., and Agate, F. J., Jr., RECENT PROG. HORMONE RES. 15, 165 (1959).
5. Midgley, A. R., Jr., and Niswender, G. D., ACTA ENDOCRINOL. Supp. 147, 320 (1970).
6. Abraham, G. E., J. CLIN. ENDOCR. 29, 866 (1969).
7. Mikhail, G., Wu, C. H., Ferin, M., and Vande Wiele, R. L., STEROIDS 15, 333 (1970).
8. Mayes, D., Furuyama, S., Kem, D. C., and Nugent, C. A., J. CLIN. ENDOCR. 30, 682 (1970).
9. Niswender, G. D., and Midgley, A. R., Jr., Abstract 22, PROGRAM OF THE 1969 MEETING OF THE ENDOCRINE SOCIETY.
10. Mayes, D., and Nugent, C. A., J. CLIN. ENDOCR. 28, 1169 (1968).
11. Mayes, D., and Nugent, C. A., STEROIDS 14, 389 (1970).
12. Erlanger, B. F., Borek, F., Bieser, S. M., and Liberman, S., J. BIOL. CHEM. 228, 713 (1957).
13. Kirshner, M. A., Lipsett, M. B., and Collins, D. R., J. CLIN. INVEST. 44, 657 (1965).
14. Kobayashi, T., Lobotsky, J., and Lloyd, C. W., J. CLIN. ENDOCRINOL. 26, 610 (1966).
15. Ito, T. and Horton, R., Abstract 74, PROGRAM OF THE 1970 MEETING OF THE ENDOCRINE SOCIETY.