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ASSAY OF TESTOSTERONE, PROGESTERONE AND 17α-HYDROXYPROGESTERONE IN HUMAN PLASMA BY RADIOIMMUNOASSAY AFTER SEPARATION ON HYDROXYALKOXYPROPYL SEPHADEX

OLLI JÄNNE, DAN APTER and REIJO VIHKO

Steroid Research Laboratory, Department of Medical Chemistry, University of Helsinki, SF-00170 Helsinki 17, and Department of Clinical Chemistry, University of Oulu, SF-90220 Oulu 22, Finland

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SUMMARY

A method for the determination of testosterone, progesterone and 17α-hydroxyprogesterone in 1–2 ml samples of male and female blood plasma is described. After extraction of the unconjugated steroids, they are chromatographed on a column of a highly lipophilic Sephadex derivative, hydroxyalkoxypropyl Sephadex, in light petroleum–chloroform, 95:5. The final measurement of the individual steroids is made by radioimmunoassay using dextran-coated charcoal in the separation of bound and unbound radioactivity.

Using 1–2 ml plasma samples the limit of sensitivity for progesterone and testosterone determination was about 0·1 ng/ml and for 17α-hydroxyprogesterone about 0·15 ng/ml.

The coefficients of variation in the determination of these steroids ranged from 7·9–15·4%. The blank values, obtained by analysis of quartz-distilled water by the method described, were negligible in the testosterone and 17α-hydroxyprogesterone determinations. Values below 10 pg were sometimes observed in the progesterone analysis.

Plasma concentrations of these three steroids in a group of females both in the follicular and luteal phase of the menstrual cycle and in connexion with the intake of oral contraceptive of the combination type are presented. In addition, values obtained in a group of normal males are also given.

The concentration values can be obtained within two days of drawing the blood samples and one technician can analyze 20 plasma samples in a 5-day period.

INTRODUCTION

The introduction of radioimmunoassay (RIA) into the field of steroid hormone analysis has greatly increased the sensitivity of measurements. It is expected that many steroid hormones circulating in blood plasma in very low concentrations may be measured in the near future by radioimmunoassay. Due to the relative nonspecificity of the antibodies used, various chromatographic purification steps are incorporated into the methods, but the usual aim is to purify a single steroid sufficiently to allow its RIA. Hydroxyalkoxypropyl Sephadex was introduced as a lipophilic support for effective chromatographic steroid separations[1] and it has been used in a method employing a competitive protein binding technique in the determination of 17α-hydroxyprogesterone[2]. In this study, chromatography on hydroxyalkoxypropyl Sephadex has been used to separate testosterone, progesterone and 17α-hydroxyprogesterone isolated from a single plasma specimen to allow their subsequent quantification by RIA using antibodies of carefully checked specifity.

Trivial and systematic names of the steroids: 5α-Dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; epitestosterone 17β-hydroxy-4-androsten-3-one.

EXPERIMENTAL

Solvents and reagents. Diethyl ether and ethyl acetate (anal. grade, Merck AG, Darmstadt, Germany) were twice distilled. Chloroform (Merck) was distilled, 1% of ethanol added and stored in a dark bottle. Light petroleum (BDH, Ltd., Poole, England) was twice distilled and a fraction boiling at 66–68°C used. Succinic anhydrine (Fluka AG, Buchs SG, Switzerland), tributylamine (BDH) isobutylchloroformate (Sigma Chemical Co., St. Louis, Mo., U.S.A.), O-(carboxymethyl)-hydroxylamine (Pfalz and Bauer, Inc., Flushing, N.Y., U.S.A.), 2-ethoxy-6,9-diaminoacridine lactate (Rivanol, University Pharmacy, Helsinki, Finland) were used as supplied.

Glassware. The glassware was not used in analyses other than RIA. Non-disposable material was first washed with detergent solution, carefully rinsed and then treated with potassium dichromate–sulphuric acid solution, rinsed with tap water and quartz-distilled water and finally with absolute ethanol.

Hydroxyalkoxypropyl Sephadex. Sephadex LH-20 was purchased from Pharmacia, Uppsala, Sweden, and hydroxyalkoxypropyl Sephades prepared as described by Ellingboe *et al.*[1]. The relative hydroxyalkoxypropyl content on a weight basis was 53·4–54%. Recently, a commercial preparation has become available (Lipidex™, Packard–Becker, B.W. Chemical Operations, Groningen, the Netherlands).

Glass columns (i.d. 6 mm) were packed with 2·5 g of hydroxyalkoxypropyl Sephadex in light petroleum–chloroform, 95:5 and the gel was allowed to settle by gravitation. Solvent was allowed to run through the column for at least 24 h before it was used for steroid separations[2]. The resulting gel column had a length of about 20 cm.

Steroids and steroid derivatives. Non-labelled steroids were obtained from Ikapharm, Ramat-Gan, Israel, or Steraloids, Inc., Pawling, N.Y., U.S.A., and were purified on hydroxyalkoxypropyl Sephadex before use.

[1,2,6,7-^{3}H]-Testosterone (S.A. 100 Ci/mmol) and [1,2,6,7-^{3}H]-progesterone (S.A. 110 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, United Kingdom, and [1,2-^{3}H]-17α-hydroxyprogesterone (S.A. 50 Ci/mmol) from New England Nuclear Corporation, Boston, Mass., U.S.A. The radioactive steroids were purified on hydroxyalkoxypropyl Sephadex at two-month intervals, about 10^7 d.p.m. at a time.

Testosterone-17β-hemisuccinate, 11α-hydroxyprogesterone-11α-hemisuccinate and 11-deoxycortisol-21-hemisuccinate were purchased from Steraloids, Inc., and used as supplied. Testosterone-3-carboxymethyl oxime was prepared essentially as described by Erlanger *et al.*[3].

Antisera. Steroid derivatives were linked to bovine serum albumin (Albumin, bovine, Sigma Chemical Co.) using the mixed anhydride reaction of Erlanger *et al.*[3]. Prior to use free fatty acids were removed from the BSA using the method of Chen[4]. The molar incorporation of the hapten per mol of BSA was estimated by U.V. spectrophotometry[3]. The hapten/BSA ratios for the antigens prepared were as follows: Testosterone-3-BSA, 36; testosterone-17-BSA, 23; 11α-hydroxyprogesterone-11-BSA, 31; and 11-deoxycortisol-21-BSA, 20.

To initiate the immunization procedures 2 mg of the antigen, dissolved in 1 ml of 0·9% aqueous sodium chloride, was emulsified in 3 ml of complete Freund's adjuvant and administered intramuscularly and subcutaneously at multiple sites to rabbits. The procedure was repeated twice at 2-week intervals. Thereafter, 1·5 mg of the antigen emulsified in a 2:1 mixture of incomplete and complete Freund's adjuvant, was injected once a month. Bleeding of the animals was started after the third injection and was repeated 7–10 days after every antigen administration. Antisera suitable for RIA were usually obtained 3 months after commencement of the treatment. They were treated with Rivanol[5] and stored in 2 ml aliquots at −20°C until dilution and use.

Saturation analysis

Phosphate buffer and dextran-coated charcoal were as described by Youssefnejadian *et al.* [6].

Radioactive steroids purified on hydroxyalkoxypropyl Sephadex were dissolved separately in the phosphate buffer to give solutions of about 20,000 d.p.m./100 μl (testosterone and progesterone, about 25 pg, and 17α-hydroxyprogesterone, about 60 pg).

Non-labelled steroids were dissolved in ethanol. The purity and concentrations of the steroids in the stock solutions were confirmed by gas–liquid chromatographic analysis of the O-methyloxime or O-methyloxime-trimethylsilyl ether derivatives of the steroids in question[7].

Antiserum dilutions in the phosphate buffer were made using the Rivanol-treated material. A dilution was chosen, which was capable of binding 35–50% of a radioactive steroid solution containing about 20,000 d.p.m.

The antiserum against testosterone-3-BSA was used at a dilution of 1:30,000, testosterone-17-BSA 1:2500, 11α-hydroxyprogesterone-11-BSA 1:250 and 11-deoxycortisol-21-BSA 1:7000.

Radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer. The volume of the buffer containing the bound radioactive steroid was 1·3 ml, to which 10 ml of the scintillator solution, Insta-Gel® (Packard Co.), was added. The samples were counted to at least a total of 10,000 counts.

Material for analysis. Blood samples were drawn from female and male volunteers without any apparent disease. Almost all were people working at these institutions. The samples were drawn before noon into glass tubes, allowed to clot and then centrifuged. The serum was stored at −20°C until analyzed.

Procedure for the determination of testosterone, progesterone and 17α-hydroxyprogesterone

A flow-sheet of the method is shown in Fig. 1. Plasma, 1–2 ml, was extracted twice with 5 ml portions of diethyl ether–ethyl acetate (1:1, by vol.) by agitating

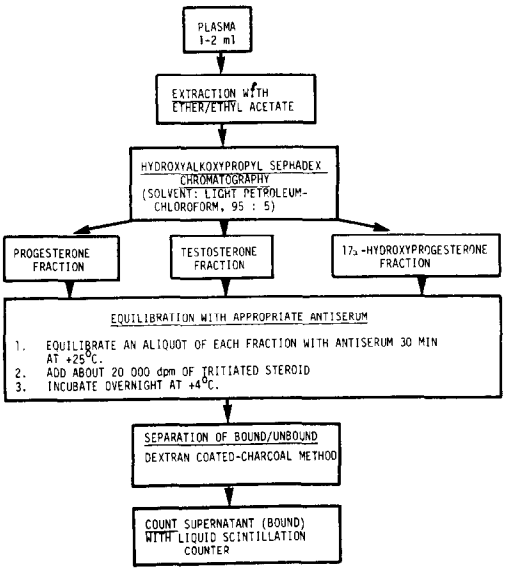

Fig. 1. Flow-sheet for the method used for the analysis of testosterone, progesterone and 17α-hydroxyprogesterone in human peripheral blood plasma.

the mixture for 1 min with a Vortex–Genie mixer (Scientific Industries, Inc., Springfield, Mass., U.S.A.). The combined solvent phases were washed twice with 1 ml of water, which in turn was subsequently extracted with 1 ml of the diethyl ether–ethyl acetate mixture. The combined extracts (12 ml) were carefully evaporated to dryness in a stream of N_2.

The residue was transferred in two 0·5 ml portions of light petroleum–chloroform, 95:5, to a hydroxyalkoxypropyl Sephadex column packed in the same solvent. Progesterone was eluted in the effluent fraction from 10–20 ml, testosterone in the fraction from 48–72 ml and 17α-hydroxyprogesterone in the fraction from 72–105 ml.

The fractions containing testosterone, progesterone and 17α-hydroxyprogesterone, respectively, were evaporated *in vacuo* and transferred in two 0·5 ml aliquots of ethanol to a small glass tube.

Table 1. Volumes of plasma and aliquots of fractions taken for assay

Source/Steroid	Volume of plasma (ml)	Aliquot for assay (%)
Women:		
Progesterone	2·0	F*: 5, 10; L: 1, 2
Testosterone	2·0	F and L: 5, 10
17αOH-Progesterone	2·0	F: 5, 10; L: 2, 5
Men:		
Progesterone	2·0	5, 10
Testosterone	2·0	1, 2
17αOH-Progesterone	2·0	2, 5

* F = follicular phase, L = luteal phase.

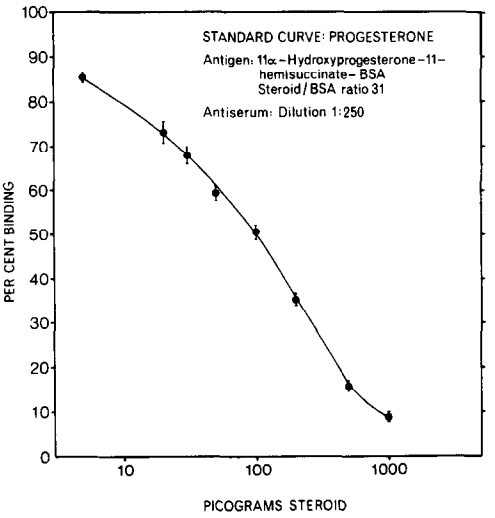

Fig. 2. Standard curve for progesterone.

For the saturation analysis 1–10% samples (10–100 μl) of the ethanol solution (Table 1) were transferred to 3 ml disposable polystyrene tubes and evaporated to dryness. For example, progesterone in the follicular phase was measured by taking duplicate 5 and 10% aliquots (Table 1). The appropriate diluted antiserum (100 μl) was added, the tube carefully agitated and allowed to stand at room temperature for 30 min. The radioactive competitor was then added in 100 μl of the buffer, the contents mixed and allowed to stand at + 4°C overnight.

The subsequent steps were performed in a cold room (+4°C). Buffer, containing 0·5% gelatin (100 μl) and 1·0

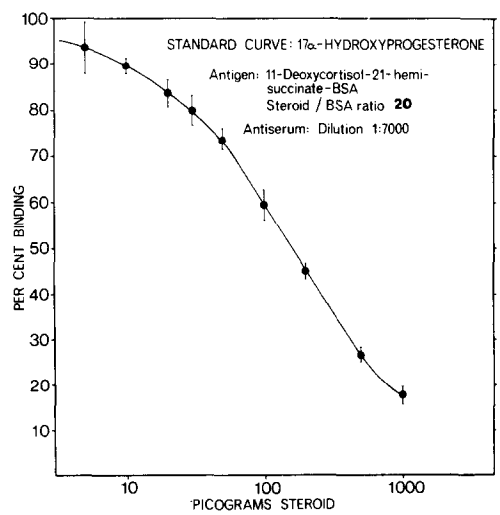

Fig. 3. Standard curve for 17α-hydroxyprogesterone.

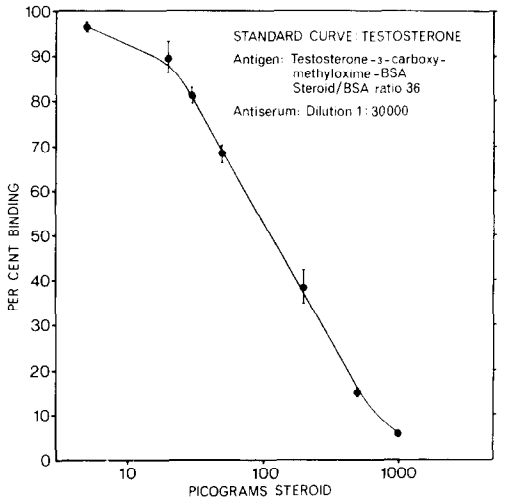

Fig. 4. Standard curve for testosterone.

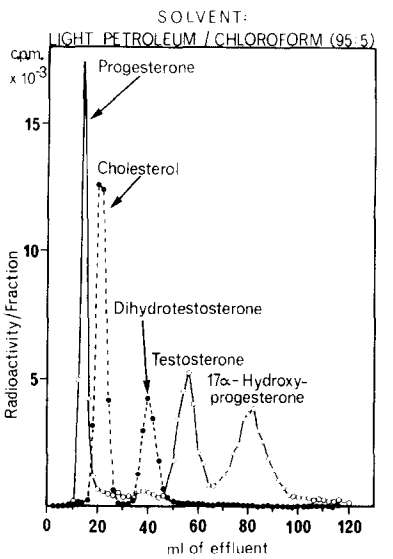

Fig. 5. Elution of certain steroids from a 2·5 g column of hydroxyalkoxypropyl Sephadex in light petroleum–chloroform, 95:5.

ml of the dextran-coated charcoal solution were added to each tube, the tubes were agitated and allowed to stand at +4°C for 15 min. The tubes were then centrifuged at +4°C for 15 min at 4000–5000 rev./min, the supernatant decanted into a counting vial, the scintillator solution added and the radioactivity measured.

Standard curves and calculation of results. Figures 2–4 represent standard curves for the determination of progesterone, 17α-hydroxyprogesterone and testosterone, respectively. All the plots shown have been constructed on the basis of 10 determinations of the standard curve.

In every series of analyses a standard curve was estimated as follows: 0, 5, 20, 50, 100, 200, 500 and 1000 pg of a steroid in question were pipetted in duplicate in 100 μl of ethanol to disposable polystyrene tubes and taken to dryness. After addition of the appropriate antiserum, the tubes for the standard curve were processed identically with those described above for the unknown sample. The amount of the steroid to be measured in the sample subjected to RIA was calculated from the appropriate standard curve.

RESULTS AND DISCUSSION

The specificity of the method based on RIA for the determination of a steroid hormone in biological specimens is dependent on the efficacy of the purification procedure and on the specificity of the antiserum used. Chromatography on a 2·5 g column of hydroxyalkoxypropyl Sephadex in light pertoleum–chloroform, 95:5, efficiently separates several unconjugated neutral steroids present in human plasma from each other (Fig. 5, Table 2). In the present investigation this separation capacity has been utilized in the determination of plasma progesterone, 17α-hydroxyprogesterone and testosterone. The respective antibodies were produced by conventional methods (see Experimental). The association constants of the antibodies were $1{\cdot}1 \times 10^{10}$ l/mol for progesterone, 7×10^{9} l/mol for 17α-hydroxyprogesterone and 7×10^{9} l/mol for testosterone at +4°C, as measured from a Scatchard-type plot[8].

The binding specificity of the antisera were checked using a number of steroids normally present in biologi-

Table 2. Relative elution volumes of some steroids on hydroxyalkoxypropyl Sephadex column (53·4%, 2·5 g). Solvent: Light petroleum (b.p. 66–68°C)–chloroform, 95:5 v/v

Compound	Elution volume
Progesterone	1·00
4-Androstene-3,17-dione	1·14
5α-Dihydrotestosterone	2·86
5β-Dihydrotestosterone	3·14
Testosterone	3·85
Epitestosterone	3·90
Dehydroepiandrosterone	4·50
5α-Pregnane-3,20-dione	0·86
5β-Pregnane-3,20-dione	0·71
20α-Hydroxy-4-pregnen-3-one	2·57
20β-Hydroxy-4-pregnen-3-one	2·36
Pregnenolone	2·86
Deoxycorticosterone	2·29
17α-Hydroxyprogesterone	5·85
17α-Hydroxypregnenolone	18·80
Aldosterone	>20
Cortisol	>20
Cholesterol	1·50

Table 3. Relative affinity of the antiserum to 11α-hydroxyprogesterone-11-hemisuccinate–BSA for some steroid ligands

Steroid	Relative affinity
Progesterone	1·00
5α-Pregnane-3,20-dione	0·864†
5β-Pregnane-3,20-dione	0·910†
20α-Hydroxy-4-pregnen-3-one	0·003
20β-Hydroxy-4-pregnen-3-one	0·002
Pregnenolone	<0·0001
17α-Hydroxyprogesterone	0·034
Deoxycorticosterone	0·015
Corticosterone	<0·001
Cortisol	<0·0001
4-Androstene-3,17-dione	<0·001†
Testosterone	0·001
Cholesterol	0†

† Eluted partly in the "Progesterone fraction" on hydroxyalkoxypropyl Sephadex.

cal samples (Tables 3–6). Regarding progesterone, 5α-pregnane-3,20-dione, 5β-pregnane-3,20-dione, 4-androstene-3,17-dione and a small part of the plasma cholesterol (Fig. 5, Table 2) are eluted in the same fraction from the column. The antiserum used in this assay has a relative affinity* for 5α-pregnane-3,20-dione and 5β-pregnane-3,20-dione close to that for progesterone (Table 3). Both these compounds are known metabolites of progesterone in humans (see 9), but data on their plasma concentrations do not seem to be available. Thus, the present method gives values for plasma progesterone, which include 5α- and 5β-pregnane-3,20-dione.

The antiserum used in the determination of 17α-hydroxyprogesterone was produced against 11-deoxycortisol-21-hemisuccinate–BSA. It did not cross-react to a significant degree with any of the steroids tested apart from 11-deoxycortisol used in the immunization

Table 4. Relative affinity of the antiserum to 11-deoxycortisol-21-hemisuccinate–BSA for some steroid ligands

Steroid	Relative affinity
17α-Hydroxyprogesterone	1·00
11-Deoxycortisol	0·712
Progesterone	0·079
5α-Pregnane-3,20-dione	0·001
5β-Pregnane-3,20-dione	<0·001
20α-Hydroxy-4-pregnen-3-one	<0·001
20β-Hydroxy-4-pregnen-3-one	<0·001
Pregnenolone	≪0·001
Deoxycorticosterone	0·022
Corticosterone	≪0·001
11α-Hydroxyprogesterone	<0·001
Cortisol	0·002
4-Androstene-3,17-dione	<0·001
Testosterone	0·013
Dehydroepiandrosterone	≪0·001
Estradiol-17β	≪0·001

* Relative affinity of the antiserum towards various ligands was measured by incubating different amounts (0·01–100 ng) of the steroid with [1,2,6,7-^{3}H]-progesterone. A semilogarithmic plot of per cent of bound radioactivity (c.p.m.) vs the log of the mass of the competing ligand added gave a competition curve for each steroid. The amount of the steroid needed to decrease the binding of [1,2,6,7-^{3}H]-progesterone by 50% was calculated from these plots. The ability to compete with tritiated progesterone for binding sites on the antiserum was then estimated relative to unlabelled progesterone. The same kind of technique was used in assessing relative affinity of the antisera used in the determination of testosterone and 17α-hydroxyprogesterone.

Table 5. Relative affinity of the antiserum to testosterone-3-carboxymethyloxime–BSA for some steroid ligands

Steroid	Relative affinity
Testosterone	1·00
4-Androstene-3,17-dione	0·027
5α-Dihydrotestosterone	0·638
5β-Dihydrotestosterone	0·500
Epitestosterone	0·003 †
Dehydroepiandrosterone	<0·001
5α-Androstane-3β,17β-diol	0·020
5-Androstene-3β,17β-diol	0·002
Progesterone	0·002
Deoxycorticosterone	<0·001
Corticosterone	≪0·001
Cortisol	0·001
Estradiol-17β	≪0·0001
Cholesterol	≪0·0001

† Elution in the "Testosterone fraction" on hydroxyalkoxypropyl Sephadex.

Table 6. Relative affinity of the antiserum to testosterone-17-hemisuccinate–BSA for some steroid ligands

Steroid	Relative affinity
Testosterone	1·00
4-Androstene-3,17-dione	0·440
5α-Dihydrotestosterone	0·182
5β-Dihydrotestosterone	0·129
Epitestosterone	0·024†
Dehydroepiandrosterone	<0·001
5α-Androstane-3β,17β-diol	<0·001
5-Androstene-3β,17β-diol	<0·001
Progesterone	0·432
11α-Hydroxyprogesterone	0·003
17α-Hydroxyprogesterone	0·018
Deoxycorticosterone	0·645
Corticosterone	0·015
Cortisol	0·003
Estradiol-17β	≪0·001
Cholesterol	≪0·001

† Elution in the "Testosterone fraction" on hydroxyalkoxypropyl Sephadex.

Table 7. Recovery of radioactive steroids (mean ± S.D., n = 6)

Steroid	Extraction: diethyl ether (2 × 8 ml)	Extraction: diethyl ether/ethyl acetate (2 × 5 ml)
Progesterone	83·9 ± 5·1%	87·8 ± 2·3%
Testosterone	79·6 ± 8·1%	79·6 ± 6·5%
17α-Hydroxy-progesterone	82·6 ± 3·3%	88·3 ± 7·7%

Table 8. Replicate analyses (8) of progesterone, 17α-hydroxyprogesterone and testosterone in female and male plasma pools

Steroid	(ng/ml) (±S.D.)	Coefficient variation (%)
Women: luteal phase		
Progesterone	13·4 ± 1·7	12·9
17α-Hydroxy-progesterone	0·71 ± 0·06	7·9
Testosterone	0·44 ± 0·07	15·2
Men:		
Progesterone	0·19 ± 0·03	13·4
17α-Hydroxy-progesterone	0·76 ± 0·11	15·4
Testosterone	9·2 ± 1·2	13·2

(Table 4). Furthermore, of the steroids so far investigated, none are eluted in the 17α-hydroxyprogesterone fraction (Table 2). Thus, the present method would seem very specific for the determination of 17α-hydroxyprogesterone.

To raise an antiserum for the determination of testosterone, two derivatives of testosterone were used, one coupled to BSA at C-3, the other at C-17. The only steroid of those tested which was eluted entirely in the same fraction as testosterone was epitestosterone (Table 2). This compound, known to be present in human plasma (see 10), reacts only slightly with the antiserum to testosterone-3-carboxymethyloxime–BSA (Table 5). Furthermore, because antisera with a higher content of antibodies to testosterone were obtained using this antigen, antiserum to testosterone-3-carboxymethyloxime–BSA was used in the present determination of testosterone in plasma. By this means, a more sensitive determination of testosterone was possible. One of the advantages of the chromatographic procedure used in this investigation is that it completely separates testosterone from 5α- and 5β-dihydrotestosterone. Thus, testosterone determination using this method seems highly specific.

The recovery of radioactive steroids added to plasma and then chromatographed on hydroxyalkoxypropyl Sephadex is shown in Table 7. Extraction with diethyl ether–ethyl acetate, 1:1 was more efficient than with diethyl ether alone. So, this solvent mixture was used in the plasma extraction. Because of the high recoveries in the extraction procedure and the subsequent chromatography, the use of internal standards was considered unnecessary.

The precision of the method was determined by repeated analyses of the three steroids in two pools of plasma. The results are summarized in Table 8. The coefficient of variation was 7·9–15·4%.

The accuracy of the method was assessed by determining the recovery of known amounts of non-radioactive steroids over a wide concentration range added to the two pools of plasma (Table 9).

The sensitivity of the method can be expressed in several ways. In the final RIA, 5 pg of progesterone or testosterone and 10 pg of 17α-hydroxyprogesterone usually gave a displacement of the appropriate bound radioactive steroid which was significantly different from the zero value (Figs. 2–4). The blank values, obtained by extracting 2 ml of quartz-distilled water and carrying the extract through the entire procedure were negligible in the testosterone and 17α-hydroxyprogesterone determinations, and usually not greater than 10 pg in the progesterone determination. Using the usual plasma volume and aliquots for final RIA

Table 9. Recovery of non-radioactive progesterone, 17α-hydroxyprogesterone and testosterone added to two pools of blood plasma

Sample/Steroid	Concentration (ng/ml)	Added (ng)	Number of analyses	Recovery (ng %)	Coefficient of variation (%)
Men:					
Progesterone	0·23	0·10	5	0·09 90	9
17α-Hydroxyprogesterone	0·65	0·15	5	0·13 88	11
		0·35	5	0·33 94	17
Testosterone	9·22	2·00	5	1·78 89	8
		6·00	5	5·41 90	14
Women:					
Progesterone	9·98	4·00	4	3·15 79	11
		13·00	4	9·53 73	4

Table 10. Mean plasma progesterone, 17α-hydroxyprogesterone and testosterone concentrations in a group of women and men (ng/ml ± S.D.)

Steroid	Women* Follicular	Women* luteal	Oral contraceptives	Men
Progesterone	0·50 ± 0·30	14·0 ± 9·61	0·34 ± 0·17	0·39 ± 0·17
range	0·20 – 0·80	4·39 – 23·61	0·17 – 0·51	0·22 – 0·56
17α-Hydroxyprogesterone	0·67 ± 0·35	1·97 ± 0·53	0·60 ± 0·26	1·08 ± 0·30
range	0·32 – 1·02	1·44 – 2·50	0·34 – 0·86	0·78 – 1·38
Testosterone	0·34 ± 0·12	0·41 ± 0·18	0·20 ± 0·07	7·42 ± 2·81
range	0·22 – 0·46	0·23 – 0·59	0·13 – 0·27	4·61 – 10·23

* Number and age of subjects: Women: Follicular phase 13; 23–33 years. Luteal phase 15; 23–40 years. On oral contraceptives 6; 24–30 years. Men: 25; 21–59 years (80% between 21 and 30 years).

(Table 1), the lowest concentration of progesterone and testosterone measurable was about 0·10 ng/ml and of 17α-hydroxyprogesterone 0·15 ng/ml. The blank values were not subtracted from the values obtained for the individual steroids.

The concentrations of progesterone, 17α-hydroxyprogesterone and testosterone determined in plasma are given in Table 10. With regard to progesterone and 17α-hydroxyprogesterone levels in the two phases of the menstrual cycle, the values obtained are very similar to those found in recent reports[11–14]. In subjects taking oral contraceptives of the combination type the concentrations of these two steroids are of the same order of magnitude as in the follicular phase (Table 10). Few reference values are available for the concentration of progesterone and 17α-hydroxyprogesterone in male plasma. The values obtained for progesterone concentration are close to those found by Yoshimi and Lipsett[15] and by Bodley *et al.*[16] but they are somewhat higher from those reported by Furuyama and Nugent[17] and Abraham *et al.*[11]. The concentration of 17α-hydroxyprogesterone in male plasma found using the present method is identical to that found by Stone *et al.*[14] using competitive protein binding after chromatography on a celite column.

For testosterone concentration in male and female plasma (Table 10) a large number of reference values are available. The values presented here for male plasma are in good agreement with those obtained by other investigators (literature up to and including 1969, see ref. 18, post-1969 see 19, 20). The values obtained for testosterone in female plasma are among the lowest published[18–20]. This is obviously due to the fact that other endogenous steroids are removed from the testosterone fraction by chromatography on hydroxyalkoxypropyl Sephadex. The difference in the mean plasma concentration of testosterone in the follicular and the luteal phase of the menstrual cycle is not significant, whereas the plasma testosterone concentration is significantly lower in the group of women taking oral contraceptives of the combination type than in women in the follicular or luteal phase not undergoing hormone treatment. It can thus be suggested that about 30–50% of the circulating testosterone or its precursor is of ovarian origin, possibly depending on the phase of the menstrual cycle. Estimates of ovarian testosterone secretion based on ovarian venous levels have indicated that from 2·5 to 20% of the blood testosterone in normal women could be secreted by the ovaries (see 21).

One technician can analyze the three steroids by this method in 20 plasma samples in a 5-day period.

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