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ROUTINE CLINICAL DETERMINATION OF TESTOSTERONE IN PLASMA BY COMPETITIVE-PROTEIN-BINDING

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SUMMARY

A method is described for the determination of testosterone in peripheral female plasma, based on a competitive protein-binding technique. The procedure includes extraction, precipitation of fatty material in cold methanol, and chromatography on partially deactivated neutral alumina. Sex steroid-binding plasma protein (SBP) is derived from late twin pregnancy plasma. The precision and practicability of the method make it suitable for clinical use. The sensitivity is 11 ng/100 ml of plasma in a duplicate determination. The method eliminates most of the steroids which interfere with the measurement of testosterone in plasma, and the results are comparable to those obtained with highly specific procedures. The mean plasma testosterone found in normal females is: 48 ng/100 ml (range 24–73) and in normal males: 740 ng/100 ml (range 430–960). Values are reported for a number of subjects with various diseases.

INTRODUCTION

A NUMBER of reports in the last seven years describe precise methods for measuring testosterone by fluorometric, double isotope derivative, and electron capture gas–liquid chromatographic techniques (for references until 1969 see [1]). However, these methods are tedious, costly and of limited value for general clinical use. For these reasons the possibility of determining testosterone in female plasma by the competitive protein-binding technique has been under intensive study during the last three years. Studies have been presented on the characteristics of sex steroid-binding plasma protein (SBP) (for references until 1970 see [2]) and determinations of testosterone by competitive protein-binding in various clinical conditions [3–14].

The relative merits of these techniques have not yet been definitely established, as evidenced by the continued research on all the above-mentioned different kinds of methods (recent studies:[15–19]). A critical evaluation introducing the concept of total figure of merit (combination of reliability and practicability) clearly shows the superiority of the binding techniques[20]. Therefore a rapid and simple method has been developed which is acceptable for clinical routine, but, despite this, retains a reasonable degree of specificity. This has been obtained by the introduction of some improvements to similar methods already in existence. These are: (1) Incorporation of precipitation of fatty material in cold methanol [21]. (2) rapid small-column chromatography and (3) use of late twin pregnancy plasma as a source of SBP[22].

EXPERIMENTAL

Radioactive steroids

The following were obtained from the Radiochemical Centre, Amersham, England: [1,2-^{3}H] testosterone, S.A. 153 mCi/mg; [1,2-^{3}H] cortisol, S.A. 93 mCi/mg; [4-^{14}C] 17α-hydroxyprogesterone, S.A. 113 mCi/mg.

The following were obtained from N.E.N. Corp., Boston, U.S.A.: [6,7-^{3}H] oestrone, S.A. 135 mCi/mg; [6,7-^{3}H] oestradiol-17β, S.A. 128 mCi/mg; [4-^{14}C] oestriol, S.A. 0,15 mCi/mg; [1,2-^{3}H] progesterone, S.A. 106 mCi/mg; [4-^{14}C] cholesterol, S.A. 0,14 mCi/mg. The purity of the radioactive steroids was verified before use by paper chromatography and scanning with a 4π Tracerlab paper scanner. For the radioactive testosterone the following chromatographic system was used: 80% aqueous methanol: ligroin b.p. 100–120°C for testosterone and its acetate.

Reference standards

Testosterone and other non-labelled steroids were purchased from Ikapharm, Ramat-Gan, Israel. Their purity was verified by gas–liquid chromatography (F & M, Avondale, Pa. U.S.A., Model 400) on a 1% SE-30 liquid phase, using the free steroid and, if the steroid contained hydroxyl group(s), the trimethyl silyl ether derivative.

Solvents

All solvents were of analytical grade. The following were redistilled once in an all-glass fractionating distillation system: ethyl acetate (Merck); ethyl ether, peroxide-free (Orion, Finland), *n*-hexane (J. T. Baker); methanol, absolute, acetone-free (Fluka).

Reagents

Florisil (magnesium silicate) 60–100 mesh (Kistner, Sweden) was washed four times with distilled water and the finer particles were decanted off. The remaining particles were dried overnight at 100°C. Sodium hydroxide puriss. (EKA, Sweden). Neutral alumina, activity grade I, for chromatography (Merck). Quartz, fine granular, washed and calcined p.a. (Merck). Water, distilled once, deionized (Resin: Amberlite MB-3) and then twice distilled in a quartz distillation system.

Radioactivity measurements

They were performed in a WALLAC DECEM-NTL 314 automatic liquid scintillation spectrometer (Wallac, Turku, Finland). The efficiency for tritium in unquenched samples was 48%, with a background of 10–12 c.p.m. in disposable polyethylene vials. The quenching effect due to the presence of biological material was corrected by the external standard channels-ratio method, the difference in counting efficiency being less than 0·5% in all experiments.

DETAILS OF THE METHOD FOR TESTOSTERONE DETERMINATION

Collection and preparation of the plasma samples

Heparinized blood is collected at 8 a.m. from fasting patients. Haemolysis should be avoided. The blood is centrifuged within 30 min of withdrawal and either processed immediately or kept at −20°C. Under these conditions the testosterone content is not appreciably altered for up to three months. If frozen plasma is used, after thawing and mixing, centrifugation is recommended before extraction.

Extraction

To 4 ml of plasma [1,2-^{3}H] testosterone in methanolic solution (20 μl, approx. 1500 c.p.m.) and 1 ml of 2N NaOH are added. Extraction is carried out with ethyl ether–ethyl acetate (1 : 1, v/v) for 10 min in a horizontal bed shaker, inside Teflon-stoppered Pyrex glass tubes (length 14 cm, dia. 2 cm). This extraction is repeated twice, each time with 8 ml of the solvent. The organic phase is transferred, after 5 min standing, to a Teflon-stoppered Pyrex glass tube (length 6·5 cm, dia. 3 cm) and washed first with 2 ml of 2N NaOH and then twice with 2,5 ml of water, shaking for 30 sec each time with a Vortex-Genie mixer. Each time, after 5 min standing, the lower phase is removed with a clean disposable Pasteur pipette and discarded. The organic solvents are then evaporated in the same tube at 45–50°C under a stream of nitrogen. The purity of the nitrogen is improved by passage of it through cotton wool and anhydrous calcium chloride.

Precipitation of fatty material in cold methanol

To the dry residue 1 ml of 70% aqueous methanol (v/v, 50°C) is added and vigorously mixed in the Vortex for 1 min. Then a further 4 ml of the same methanolic solution is added, the walls of the tube being cleaned with the solvent, and the tube is kept overnight or longer at −20°C. It is centrifuged in the cold (−15°C) at 6000 rev./min for 45 min. The clear supernatant is decanted off and evaporated at 45–50°C under a stream of nitrogen and then subjected to column chromatography.

Column chromatography

This is performed in a small glass column (dia. 1 cm) ending in a capillary (i.d. 0·35 mm)—as described by Adlercreutz and Schauman [23]. Three grams of neutral alumina, activity grade I deactivated with 1·5% of water, resulting in activity grade II/III according to Brockman, is prepared in *n*-hexane saturated with water, and washed with ten ml of *n*-hexane. The dry extract dissolved in *n*-hexane is transferred to the column and the tube is washed with the first eluant. The following solutions are used for elution:

(A) 13 ml of 3% methanol in *n*-hexane (v/v)
(B) 15 ml of 5% methanol in *n*-hexane (v/v)
(C) 10 ml of 7% methanol in *n*-hexane (v/v).

The *n*-hexane used in all these solutions is saturated with quartz-distilled water. The speed of chromatography is set to be 1·4–0·8 ml/min. It is important that the flow rate should be rigorously calibrated. Fast columns may be made slower by gently tapping the column. Testosterone is eluted in fraction (B). It is collected in a 15-ml conical graduated tube, and the solvent reduced to a volume of 10 ml under a stream of nitrogen. Then 1 ml is taken for determination of recovery and from the rest a suitable sample is evaporated to dryness and subjected to competitive protein-binding assay.

Each new batch of neutral alumina is standardized with radioactive cholesterol, testosterone and oestrone as markers during column chromatography. The volumes of the eluting solutions are optimized. At least 90% of the testosterone should occur in fraction (B). Not more than 5% of the cholesterol and no oestrone should occur in the testosterone (B) fraction. As a routine precaution, fractions

(A) and (C) are not discarded until recovery has been verified to be satisfactory. If it is not, their content of radioactive testosterone is checked.

Competitive protein-binding assay

To the dry residue 20 μl of methanol is added and the tubes are swirled so that the dry residue is wetted. One ml of the dilute protein with the radioactive testosterone (0·5% of late twin pregnancy plasma with testosterone binding capacity of more than $6{\cdot}5 \times 10^{-7}$ mol/l determined by a Scatchard type plot as described previously (22) in water containing approximately 40.000 c.p.m) is added to the tubes with an Eppendorf pipette. It is mixed for 60 sec with the Vortex and left for 30 min in ice water. 40 mg of Florisil is added with a plastic weighing spoon, the contents of the tubes mixed again for 30 sec with the Vortex and left in ice water for precisely 10 min. Two aliquots of 0·2 ml of the supernatant are pipetted with a constriction micropipette (Carlsberg) into separate disposable counting vials, 10 ml of Bray's scintillation mixture (naphthalene 60·0 g, PPO 4·0 g, POPOP 0·2 g, methanol 100 ml, ethylene-glycol 20 ml, dioxan ad 1000 ml) is added and the radioactivity is measured. The results are compared with the standard curve obtained under the same conditions for 0–6 ng of testosterone. Standard curves obtained with late twin pregnancy plasma have been presented in a previous publication [24].

Practical aspects

Late twin pregnancy plasma has been shown to retain its binding properties when kept for up to six months at −20°C. Equilibration of the diluted plasma with the radioactive steroid is attained overnight at +4°C. The dry residues of the evaporated extracts following chromatography are well preserved after storage at +4°C for one week. An equilibration time of four to six hours is recommended before radioactive counting. The counting time is 10 min for the sample and 30 min for the recovery sample. This counting time results in an error less than 3%. Glassware is cleaned in the way normally used in this laboratory for gas-chromatographic routine work plus an additional rinse with *n*-hexane prior to use. In a five-day week a technician can perform the analysis of approximately 35 samples. This includes all preparatory work, such as distillation of solvents, except cleaning of glassware.

The determination is performed in patients receiving no medication. This precaution is taken because a number of drugs have been shown to compete with testosterone *in vitro* [12, 25]. But even for substances that do not themselves compete *in vitro* it is not certain that this applies to their *in vivo* metabolites. Various *in vivo* radioisotope tests are also avoided before testosterone determination. Finally diurnal variation, if any [26], is obviated by using blood withdrawn at 8 a.m. and conversion to androstenedione [27] is largely avoided by using non-haemolysed samples.

RESULTS

Accuracy

The total recovery of testosterone was 73 ± 5%. The recovery step by step was as follows: Recovery during extraction was 94% (a third extraction with the same solvents gave results only 2–3% higher); recovery after washing with NaOH and water was 88%; after precipitation of fatty material in cold methanol, 81%.

Four ng of pure testosterone were added to normal female plasma (eight samples), and the mean recovery of the added testosterone was $3{\cdot}9 \pm 0{\cdot}3$ ng when corrections were made for losses during the procedure.

Specificity

The behaviour of various steroids during this procedure has been investigated, with the following results: only 2·5–4·5% of cholesterol is extracted from plasma, and of this amount 40–60% is eliminated in the precipitate formed in cold methanol. 95% of the remaining cholesterol is removed during chromatography. The results are based on multiple determinations by a routine colorimetric method[28] and after addition of a radioactive tracer, and on two determinations by gas-liquid chromatography. Results were obtained at normal and pathological levels of plasma cholesterol and with samples to which pure cholesterol had been added. Oestrogens are eliminated partly during the extraction of the plasma and partly during washing with alkali (remaining: oestrone 17%, oestradiol 10%). All the remaining oestrogens are completely eliminated during chromatography, as judged by the determination after addition of either radioactive tracers or pure reference oestrogens to the plasma and measurement of the recovery by liquid scintillation counting or gas–liquid chromatography. Although 45% of the cortisol is extracted, this steroid is completely removed during the chromatographic step. Results were obtained by the same tracer technique as was used for the other steroids and also by a fluorometric method[29]. The results of the chromatographic behaviour of other steroids shown to be present in smaller amounts in plasma are shown in Table 1. The specificity of the methods was checked in another way by the following additional experiments: a sample of normal female plasma subjected to the normal procedure and then chromatographed a second time gave the same results: With one chromatography, 37–41 ng/100 ml, with two chromatographies, 35–39 ng/100 ml (duplicate determinations). Four normal male plasma samples subjected to the normal procedure and additionally to chromatography on paper saturated with 80% aqueous methanol at 25°C in ligroin b.p. 100–110°C gave the following results: normal procedure, 440, 425, 700, 650; after paper chromatography, 400, 400, 660 and 680 ng/100

Table 1. Percentages of various steroids still present in the testosterone fraction after one chromatography cn partially deactivated alumina

1	5α-Dihydrotestosterone	50%
2	5α-Androstane-3β,17β-diol	1%
3	5α-Androstane-3α,17β-diol	3·5%
4	Androstenediol (5-Androstene-3β,17β-diol)	7%
5	Etiocholanolone	20%
6	Dehydroepiandrosterone	15%
7	Androsterone	4%
8	Androstenedione	25%
9	Cortisol	< 0·1%
10	Progesterone	3%
11	17α-Hydroxyprogesterone	50%
12	Pregnanediol	6%
13	Pregnanetriol	< 1%
14	Oestradiol-17β	0·0%
15	Oestrone	0·0%

ml, respectively. Four samples of plasma obtained at operation from two women (patients A and B) from their left (LOV) and right ovarian veins (ROV) (diagnosis: virilism) gave the following results by the present method: 955 (A, LOV), 1000 (A, ROV), 1035 (B, LOV), 1000 (B, ROV) ng/100 ml. The same samples analysed by a gas–liquid chromatographic method (Dr. T. Laatikainen, personal communication) were shown to have a concentration of 500–1000 ng/100 ml. The determination was made with a hydrogen flame detector at the lowest limit of g.l.c. detection and a more exact value could not be obtained.

Testosterone has been determined by the present method in a pooled sample of normal female plasma obtained from blood donors and the value found was 38 ± 5 ng/100 ml (eight determinations). The same sample has been analysed by a specific electron-capture gas-chromatography method[30] and the mean value found, in a duplicate determination, was 45 ng/100 ml. This result indicates that the method is specific for testosterone at low concentrations, a factor which is especially important for a method which is to be used for clinical investigations.

Non-specific blank

The value of the non-specific blank (4 ml of water treated as plasma) was constant and in the range of 0·3–0·6 ng/sample, i.e. 8–15 ng/100 ml. This value was subtracted from the sample value. Two blanks were always processed with every batch of samples. The contribution of each solvent to the blank value was tested before use, and any that gave unsatisfactory results were redistilled. With non-distilled solvents or solvents kept for more than a month or ether kept for one week the blank values obtained exceeded 50–60 ng/100 ml. Occasionally, owing to repair of the central water distillation system, extremely high blank values were encountered for a period of several weeks. The distilled water showed high conductivity and it is likely that these high blank values were due to interference with heavy metals.

Precision

The precision (s) of the method can be expressed as estimates of the SD (s) of the differences between the two results of duplicate determinations in a series of experiments. These calculations were carried out at three different testosterone levels and the results are shown in Table 2.

Table 2. Estimates of precision, expressed as estimates of the SD (s) of the differences between two results of duplicate determinations, including the coefficient of variation

Testosterone concentration (ng/100 ml of plasma)	Number of duplicate determinations used for calculating s	s ng/100 ml of plasma	Coefficient of variation (%)
0–100	19	6·9	11·4
100–500	19	22·7	9·1
500–1000	15	73·1	9·1

Sensitivity

The sensitivity of the method in a duplicate ($n = 2$) assay can be calculated from the precision (s) of the determination at levels below 100 ng/100 ml:

$$\text{Sensitivity} = \frac{t \times s}{\sqrt{n}}$$

where t is the tabulated t-value for N-1 degrees of freedom (N = the number of duplicate determinations used for calculation of s). Thus the smallest amount which differs significantly ($P < 0{\cdot}05$) from zero in a duplicate determination is 10·3 ng/100 ml.

Calculations of testosterone concentration

The actual concentration of testosterone in ng/100 ml was derived from the following equation:

$$(\text{Unknown-blank}) \times \frac{100}{\text{Recovery \%}} \times \frac{100}{\text{Volume of sample}} \times \frac{10}{\text{ml of final extract used for assay}}.$$

The amount of [^{3}H] testosterone initially added for correction of losses was not taken into account[31] since the amount left in the assay tube is insignificant (6 pg).

DISCUSSION

During the development of the present method several solvents were tested as extractants (ethyl ether, dichloromethane, ethyl acetate), and also the effect of adding sodium chloride to plasma. The technique adopted (ether–ethyl acetate) for extraction from alkalinized plasma has the following advantages: (1) the recovery of testosterone is satisfactory; (2) addition of alkali to the plasma reduces the amount of solids obtained after evaporation of the extract[32]; (3) only 2·5–4·5% of the cholesterol is extracted. Oestrogens are partly eliminated during the extraction, because they remain to a great extent in the aqueous phase. However, small but significant amounts of oestrogens are extracted, although some are removed by the subsequent alkaline wash. Finally, the remaining oestrogens are completely eliminated in the alumina chromatography. In some of the previous methods no satisfactory way of eliminating oestrogens has been included and this is a point that must be very carefully considered, owing to the significant interference of oestrogens (especially oestradiol) in the final binding assay. The solvents chosen are comparatively non-toxic, evaporate readily and seldom form emulsions.

The cold methanol treatment was found to improve the results. Ismail[13] noted better chromatographic separation after a similar step. We have preferred the method of Zander and Simmer[21] used previously in this laboratory in other determinations (for bile, amniotic fluid) because we wished to avoid the introduction of Ca^{++} (as in the method of Ismail). The precipitation of fatty material in cold methanol is technically simple to carry out and removes a significant part of the cholesterol content, which seemed essential[24]. The elimination of cholesterol (i.e. including the elimination in the following chromatographic step) was almost complete. In addition, in our hands the procedure adopted gave a higher recovery for testosterone than the technique of Ismail.

The incorporation of a chromatographic step is in accordance with the latest published methods. Some of the techniques reported[9, 12] do not include such

a step and hence the testosterone values they yield are too high. The reason is evidently failure to eliminate a number of other steroids which have been demonstrated to compete with testosterone for SBP-binding sites. The column chromatography adopted combines high practicability with a very low contribution to the blank value. Thin-layer or paper chromatography results in high blank values [3]. For the latter, in order to obtain low blanks, 2–3 weeks cleaning the paper with spectrograde methanol has proved necessary [5].

The experimental results on the affinity of various steroids for SBP – as shown in Table 3 – indicate a discrepancy which may sometimes be great. This has many causes. First, these studies were made with dilute plasma from different sources and the solutions contain variable amounts of other plasma proteins. Unequivocal determination of the affinity of each steroid will be obtained only when pure SBP is available and the exact equilibrium constants of the steroids can be determined. Second, the experimental conditions, such as steroid concentration, medium, temperature and time of incubation, were not identical. When multiple protein equilibria are involved and complex biological materials tending to distort the binding process are present, the interference of the various steroids should be assessed at the concentrations at which they are usually present in the final extract and in the exact experimental conditions of the assay. They were determined in this way in the present investigation (Table 3), i.e. in the amounts in which they are usually present in the final assay. These levels of the various steroids were calculated from the known concentrations in the plasma of female subjects reported in previous studies from other laboratories and from procedural elimination as judged from recovery experiments with radioactive and non-radioactive reference steroids.

In our assay, the source of SBP is late twin pregnancy plasma. This has proved to be the richest source of this specific binding protein [22]. and so increases the specificity of the method. (Method specificity is the sum of the individual specificities of isolation, separation and detection of a compound [33]). The temperature and time of incubation (0°C, 30 min) are favourable for the binding, and the Florisil used is convenient and has not been shown to alter the conformation of the protein(s).

Our method has been used in routine clinical laboratory analyses in various endocrinological disorders, and some of the results obtained in normal and various pathological cases are presented in Tables 4–6. The values are in good agreement with previously published results obtained with highly specific procedures [34] and correlate well with the clinical diagnosis of the patients. No attempt was made to achieve absolute specificity for testosterone. One more chromatographic step (for example a Bush B_3 or B_A system) would have been necessary to eliminate the dihydrotestosterone completely, but this would have meant a considerable reduction in the practicability, precision and sensitivity of the method.

It can be concluded from Tables 1 and 3 that the interference of non-androgenic steroids (even those present in plasma in large amounts) such as progesterone, cortisol, pregnanediol, and pregnanetriol is nil. The same is true of oestrogens, which are completely eliminated in the procedure. The interference of the three weak androgens, androsterone, etiocholanolone and dehydroepiandrosterone, is also negligible. Androstenedione, a more potent androgen and a precursor of testosterone, does not alter the results, because, despite the fact that the alumina chromatography eliminates only 75% of it, it only competes to an

Table 3. Competition relative to testosterone for binding sites in plasma (testosterone = 100) of various steroids identified or possibly present in female plasma. Concentration in female plasma, if known

	STEROID	Competition according to literature								Present study	Concentration of the free steroid in female plasma (ng/100 ml)
		3	4	6	7	8	12	35	36		
1	Dihydrotestosterone	200			100	187	285	340	300	300	17 ± 4 (Refs. 37–39)
2	5α-Androstane-3β,17β-diol					120		250	160	200	Not found in normal cases. Present as sulphate in uremia and after alcohol consumption. *Ann. Med. Fenn.* **47** (1964) 151 and *Biophys. Biochem. Acta* **152** (1968) 233.
3	5α-Androstane-3α,17β-diol	77		100	120	108		200	160	125	Normally as sulphate. *Clin. Chim. Acta* **23** (1969) 405.
4	5-Androstene-3β,17β-diol	77			90	71	65	130	50	140	Normally as sulphate
5	Etiocholanolone	< 2		15·20		0·2	0	0·1	< 1	0	300 *J. Clin. Invest.* **44** (1965) 657
6	Dehydroepiandrosterone	5	8	10·15		3·7	2		< 5	3	1040 ± 380 *J. Clin. Invest.* **44** (1965) 657
7	Androsterone	< 2		15·20	< 5	1·6	1	1	< 5	1	150 − 250 *J. Clin. Invest.* **44** (1965) 657
8	Androstenedione	< 2	2	15·20	< 5	0·3	4	1·4	< 1	2	150
9	Cortisol	< 2			< 5		0		< 3	< 1	15,000
10	Progesterone	< 2	7		< 5		0		< 5	< 1	1000 Luteal phase
11	17α-Hydroxyprogesterone		5		< 5		0		< 5	< 1	174 ± 46 Luteal phase
12	Pregnanediol	< 2			< 5					0	1000, as conjugate
13	Pregnanetriol	< 2								0	1300, as conjugate
14	Oestradiol-17β	30		80·90		10	30	60	60	60	20 Luteal phase. *J. Clin. Endocr.* **29** (1969) 149.

Note 1. The plasma values of the different steroids are approximate mean figures presented in recent literature. Some of them may be found in *Hormones in Blood* by Gray and Bacharach, Academic Press, 1967, or *Adrenal Steroids and Disease* by C. L. Cope, Pitman 1965. Some references to be found in the table.

Note. 2. The numbers heading the columns of the competition of various steroids according to the literature refer to the references of this communication.

Table 4. Testosterone levels in male patients

	Subject	Age	Testosterone (ng/100 ml)	Diagnosis
1	S.R.M.	24	714	Addison's disease
2	R.K.O.	32	25	Eunuchoidism
3	K.H.A.	44	41	Klinefelter's syndrome (XXY)
4	S.N.J.	43	36	Klinefelter's syndrome (XXY) Alcohol abuse
5	R.T.	41	33	Hypogonadism following castration
6	R.H.	17	60	Hypogonadism, gonadal dysgenesis
7	L.J.	30	1230	Acne conglobata
8	H.H.	19	1290	Acne cystica
9	M.V.	39	81	Acromegaly. Hypophysectomized
10	K.V.	28	92	Hypogonadism

Table 5. Testosterone levels in female patients

	Subject	Age	Testosterone ng/100 ml	Diagnosis – Remarks
1	A.M.	52	1450, 1430	Ovarian gynandroblastoma (Twice determined in a six months interval)
	A.M.	52	47, 54	After operation (radical extirpation). Ten days and six months after operation
2	V.S.E.	48	97	Virilism. Ovarian fibroma. Luteal cyst
	V.S.E.	48	6	Two months after operation. Total removal of uterus and adnexae
3	R.L.L.	24	155	Hirsutism, Amenorrhoea
4	T.H.M.	32	82	Hirsutism. Normal findings in laparoscopy of the ovaries
5	S.E.L.	32	82	Hirsutism
6	P.K.	20	120	Hirsutism, Oligomenorrhoea
7	K.R.	41	120	Hirsutism, Obesity. In laparoscopy right ovary enlarged
8	O.M.L.	39	70	Hirsutism, Obesity
9	L.R.	36	68	Hirsutism, Mild obesity
10	T.G.	24	56	Hirsutism, Algomenorrhoea. Irregular menstruation
11	M.R.	34	60	Hirsutism, Obesity
12	S.P.	37	90	Hirsutism
13	L.A.	34	277	Hirsutism, Obesity. Ovarian sclerosis
14	P.A.H.	35	254	Congenital adrenal hyperplasia
15	G.M.	20	200	Morgagni Syndrome
16	M.S.S.M.	20	207	Congenital adrenal hyperplasia
17	V.M.	16	280	Virilism. Secondary amenorrhoea
18	K.I.	38	420	Adrenal virilism
19	T.T.T.	25	300	Stein–Leventhal syndrome
20	P.T.	32	45	Pheochromocytoma

extent of 2%. Serious competition in the binding assay is offered by steroids with a 17β-hydroxyl group (Nos. 1, 2, 3 and 4 in Tables 1 and 3). In fact, a comparison of the binding affinities obtained in the present and the previous studies shows that our results are close to those of Murphy and Vermeulen[35, 36], i.e. that there is

a higher binding affinity for these steroids than for testosterone itself. The physiological significance of this phenomenon is not known.

The interference in the present testosterone determination method due to these three 17β-hydroxyl-containing steroids (Nos. 2, 3 and 4 in Tables 1 and 3) does not seriously invalidate the specificity of the procedure, because the plasma content of the free steroids is relatively low and they are largely (more than 90%) eliminated in the chromatographic step. As already mentioned, dihydrotestosterone is not completely eliminated in the procedure. This very important androgen has lately attracted more attention. Recently its presence in plasma has been confirmed by mass-spectrometry[37] and its levels have been determined by competitive protein-binding techniques[38, 39]. The inclusion of this compound in the present assay does not seem to reduce the value of the method for routine clinical purposes. By not excluding it completely, we are tempted to suppose that we achieve a better idea of the endocrinological status. Whereas in males the plasma testosterone level is usually a good index of its production rate, in females this is not always the case[40]. The complexities of androgen metabolism in women warrant special consideration. Other factors, such as the percentage binding to protein[41] and the clearance of steroids, play significant roles. But the assay of plasma testosterone is in any case a much better diagnostic tool than the determination of urinary total 17-ketosteroids or their fractionation.

Table 6. Plasma testosterone levels in healthy subjects

	Subjects	Number	Plasma testosterone (ng/100 ml) Mean	Range
1	Adult males	20	740	430–960
2	Adult females	20	48	24–73
3	Prepubertal boys	15	16	0–36

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REFERENCES

1. H. J. van der Molen: In *The Androgens of the Testis*, (Edited by K. B. Eik-Nes). Marcel Dekker, New York (1970) p. 145; pp. 206–215.
2. O. Steeno: *Testosterone Binding in Human Plasma*. Acco. Leuven (1970).
3. D. Mayes and C. A. Nugent: *J. clin. Endocr.* **28** (1968) 1169.
4. M. C. Hallberg, E. M. Zorn and R. G. Wieland: *Steroids* **12** (1968) 241.
5. T. Kato and R. Horton: *Steroids* **12** (1968) 631.
6. R. Maeda, M. Okamoto, L. C. Wegienka and P. H. Forsham: *Steroids* **13** (1969) 83.
7. J. Frick and F. A. Kincl: *Steroids* **13** (1969) 495.
8. R. L. Rosenfield, W. R. Eberlein and A. M. Bongiovanni: *J. clin. Endocr.* **29** (1969) 854.
9. S. Kamitsuna and V. W. Cole: Hirosh. *J. Med. Sci.* **18** (1969) 1.

10. J. A. Demetriou and F. G. Austin: *Clin. Chem.* **16** (1970) 111.
11. Von A. Uettwiller: *Z. Klin. Chem.* **8** (1970) 225.
12. D. C. Anderson: *Clin. Chim. Acta* **29** (1970) 513.
13. A. A. A. Ismail, D. N. Love and E. Nieschlag: In *Research on Steroids*, (Edited by M. Finkelstein, C. Conti, A. Klopper and C. Cassano), Pergamon Press, Oxford–New York (1970) Vol. 4, p. 311.
14. J. S. D. Winter and D. R. Grant: *Anal. Biochem.* **40** (1971) 440.
15. M. A. Kirschner and J. Taylor: *Anal. Biochem.* **30** (1969) 346.
16. C. Eaborn, C. A. Holder, D. R. M. Walton and B. S. Thomas: *J. Chem. Soc. C*, (1969) 2502
17. B. S. Thomas: J. Chromatography **56** (1971) 37.
18. W. Eechaute, G. Demeester and I. Leusen: *Steroids* **16** (1970) 277.
19. M. Sparagana: *J. Nucl. Med.* **12** (1971) 253.
20. See Ref. 1. p. 201.
21. J. Zander and H. Simmer: *Klin. Wschr.* **32** (1954) 529.
22. A. Dessypris, T. Luukkainen and H. Adlercreutz: *J. Steroid Biochem.* **1** (1970) 77
23. H. Adlercreutz and K.-O. Schauman: In *Methods of Hormone Analysis* (Edited by Breuer/Krüskemper). Georg Thieme Verlag, Stuttgart (In press).
24. A. G. Dessypris: *J. Steroid Biochem.* **1** (1970) 185.
25. B. E. P. Murphy: *Steroids* **16** (1970) 791.
26. R. Crafts, L. A. Llerena, A. Guevara, J. Lobotsky and C. W. Lloyd: *Steroids* **12** (1968) 151.
27. A. O. Brinkmann, E. Muller and H. J. van der Molen: see Ref. 13, p. 91.
28. S. Pearson, S. Stern and T. H. MacGolvack: *Anal. Chem.* **25** (1953) 813.
29. D. Mattingly: *J. Clin. Pathol.* **15** (1962) 374.
30. L. Lakshmi Kumari, W. P. Collins and I. F. Sommerville: *J. Chromatography* **41** (1969) 22.
31. See Ref. 1. p. 171.
32. A. Ogata and S. Hirano: *Proc. Imp. Acad.* **9** (1934) 349.
33. See Ref. 1. p. 180.
34. H. M. Gandy and R. E. Peterson: *J. Clin Endocr.* **28** (1968) 949.
35. B. E. P. Murphy: In *Recent Progress in Hormone Research*, (Edited by E. B. Astwood). Academic Press, New York (1969) Vol. 25, p. 591.
36. A. Vermeulen and L. Verdonck: *Acta endocr.* **64** Suppl. 147 (1970) 239.
37. V. K. Ganjam, B. E. P. Murphy, T. H. Chan and P. A. Currie: *J. Steroid Biochem.* **2** (1971) 155.
38. R. R. Tremblay, I. Z. Beitins, A. Kowarski and C. J. Migeon: *Steroids* **16** (1970) 29.
39. T. Ito and R. Horton: *J. Clin. Endocr.* **31** (1970) 362.
40. C. W. Bardin and J. A. Mahoudeau: *Ann. Clin. Res.* **2** (1970) 251.
41. O. Crépy, H. P. Klotz, D. Jouin-Courrier, M. A. Ducret and A. Schwob: *Ann. Endocr. (Paris)* **31** (1970) 437.