

Fluorimetric method for simultaneous estimation of cortisol, corticosterone, and testosterone in plasma

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SUMMARY The simultaneous estimation of steroids in plasma was carried out by the assay of cortisol, corticosterone, and testosterone. The method entails separation by means of thin layer chromatography, followed by conversion to a fluorophore and fluorimetric measurement. Its major advantages are its high specificity, its ability to detect unknown substances, and the ease with which it can be performed. The method has acceptable levels of accuracy and precision and the normal values obtained by it compare well with those given by methods in general use.

Numerous methods have been described for the determination of steroids in blood based on fluorimetry, radioimmunoassay (RIA), competitive protein binding (CPB), high performance liquid chromatography (HPLC), or enzyme immunoassay (EIA). Most of them, however, have the disadvantage that their specificities are not absolute because a certain amount of interference or cross-reactivity with other steroids may occur. This can be a major drawback when the configuration of the substance under study is in doubt.

We developed a method involving thin layer chromatography (TLC), followed by conversion to a fluorophore and fluorimetric detection, in which individual steroids are completely separated and are measured simultaneously. This is a modification and extension of the method first described by Hamman and Martin in 1969¹ for the assay of cortisol and corticosterone. The present paper is restricted to the validation of the method for the simultaneous assay of cortisol, corticosterone, and testosterone, but there is no doubt that many other steroids can be measured in this way.

Material and methods

The following material, obtained from BDH, UK, was used: benzene AR; methanol AR; methylene chloride (reagent grade) purified in the laboratory²; sulphuric acid. Steroid standards were purchased from Sigma Chemicals, Poole, Dorset.

SPECIAL EQUIPMENT

1 *Glass tubes with ground glass stoppers*, volume 19 ml,

treated with a silicone solution to reduce absorption of steroids on glass were used. All glassware should be cleaned with a non-fluorescent detergent such as Decon 90 (Decon Laboratories Ltd, Hove, Sussex).

2 *Spectrophotofluorimeter* with TLC scanning attachment (Aminco-Bowman 4-8911E, VA Howes, London) was adapted by us so as to increase the sensitivity. In the unmodified instrument the fluorescence emitted from the steroid spot on the layer passes through the glass plate before collection by the fibre optic. This arrangement was changed so that the fibre optic was positioned on the same side of the plate as the incident light, and at an angle of 45° to it. Thus the emitted fluorescence was collected directly from the surface of the layer without passing through the plate. The excitation slit width is 0.5 mm and the photomultiplier slit width is 1.0 mm. The wavelength of the exciting light is 470 nm, and that of the emitted light is 520 nm when cortisol, corticosterone, and testosterone are under study. The wavelengths may need to be varied slightly for other steroids.

3 *Chart recorder* (Bryan model No 24000 A3, VA Howes, London) was used.

4 *Glass plates* for thin layer chromatography measured 20 × 5 × 0.3 cm. These are spread with silica gel (MN Kieselgel G-HR, Camlab, Cambridge) to produce a layer with a thickness of 200 μm. Five lanes 4 mm wide are scribed into the layer along the length of the plate so that four specimens and a standard can be run together. Additional lines are scribed close to the sides of the plate to minimise edge evaporation effects. A horizontal line is scribed across the plate at the required run distance (usually 14.7 cm).

5 *The glass chromatography jar* measured 30 × 10 cm.

6 *A planimeter* (Allbrit, WF Stanley, London) for measuring area under curve on scan trace was also used.

Blood is taken into standard lithium heparin tubes (Searle Pharmaceuticals, High Wycombe, Buckinghamshire) and centrifuged immediately. The plasma is either used at once or deep-frozen until use. Plasma (2 ml) is pipetted on to 15 ml of methylene chloride in a stoppered tube of 19 ml volume. The tube is rotated horizontally for 20 minutes to extract the steroids into the solvent.² The plasma layer is aspirated and discarded. A 13 ml aliquot of the solvent phase is transferred to a clean tube and evaporated to dryness in a water bath at 50°C, under a stream of nitrogen (99.99% pure, British Oxygen Company, UK).

The residue in the tube is dissolved with three drops of methylene chloride which is spotted on to a lane on the plate at a distance of 3 cm from the bottom of the layer. The tube is further washed with two drops and then with one drop of methylene chloride which are added to the spot on the plate. During this procedure the chromatography plate is placed on a warmed metal surface to accelerate evaporation of the spotting solvent.

The lower end of the TLC plate is immersed to a depth of 2 cm in the running solvent, benzene-methanol (50:5), in the chromatography jar. The solvent is allowed to ascend to the horizontal line scribed in the layer and the plate is then dried in a current of air. This running procedure is repeated a second time to produce a better separation of the steroid spots. Fluorescence is induced by spraying the plate with 20% sulphuric acid in water, using a pressurised cannister to create a fine mist, until the layer is visibly and evenly damp all over.

The plate is then heated on a metal block in a hot air oven at 80°C for 30 minutes. After heating, the plate is cooled in air for 15 minutes to allow the fluorescence to develop, then placed layer downward in the TLC scanner. Each lane in turn is passed above the slit of exciting light and the fluorescence emitted is recorded as a series of peaks on paper. The scanning of five lanes should be completed within 20 minutes, during which time the fluorescence remains constant. The area under the peak on the scan trace is measured by means of a planimeter.

A mixture of standard steroids in water (2 ml) containing cortisol 607.2 nmol/l (22 µg/100 ml), corticosterone 57.8 nmol/l (2.0 µg/100 ml), and testosterone 17.4 nmol/l (500 ng/100 ml) is taken through the method alongside the plasmas under investigation. The testosterone concentration was reduced to 1.73 nmol/l (50 ng/100 ml) if female plasmas were being assayed. Estimations can be completed within one working day.

Normal values for cortisol, corticosterone, and testosterone were obtained from members of the hospital staff and adult hospital patients. Patients with known endocrine, liver, or renal disease were

excluded, and none of the patients was acutely ill at the time or being subjected to stressful procedures. Blood was taken in the morning between 0800–1000 hours to avoid variations due to circadian rhythm.

Results

RELATIVE FRACTION (RF) VALUES

As we were particularly concerned with cortisol, corticosterone, and testosterone the running solvent benzene-methanol (50:5) was chosen for the good separation it gave with these steroids, but it is possible to measure the concentration of other fluorogenic steroids with intermediate RF values at the same time (table 1). A typical record of the scan for cortisol, corticosterone, and testosterone is shown in fig 1. A running solvent such as chloroform-acetone-water (42:18:0.5) can be used to give a better separation of steroids with lower RF values. When steroids with an RF value of more than 0.6 are under investigation defatting of the plasma may be necessary to produce a stable baseline.³

DEVELOPMENT OF FLUORESCENCE

Most steroids do not fluoresce naturally but many will produce fluorophores when reacted with strong acids or alkalis. For the steroids under investigation here the most satisfactory method is to spray the plates with 20% sulphuric acid, followed by heating in a hot air oven at 80°C for 30 minutes. The temperature of the oven and the time for which heating is carried out were chosen to give maximum fluorescence from cortisol, corticosterone, and testosterone. The optimum temperature and time of heating, however, do vary from

Table 1 Steroid RF values in running solvent benzene:methanol (50:5)

6 β-Hydroxycortisol	0.11
20 β-Hydroxycortisol	0.15
Prednisolone	0.15
18-Hydroxycorticosterone	0.16
20 β-Hydroxycorticosterone	0.16
Oestriol	0.16
Cortisol	0.21*
Aldosterone	0.25
18-Hydroxy-deoxycorticosterone	0.33
21-Deoxycortisol	0.36
Corticosterone	0.38*
Oestradiol	0.45
Testosterone	0.57*
11-Deoxycortisol	0.68
11-Deoxycorticosterone	0.71
Oestrone	0.73
Dehydro-epiandrosterone	0.74
5 α-Dihydrotestosterone	0.74
Pregnenolone	0.78
Androstenedione	0.85
Cholesterol	0.89
Spironolactone	0.91
Progesterone	0.94

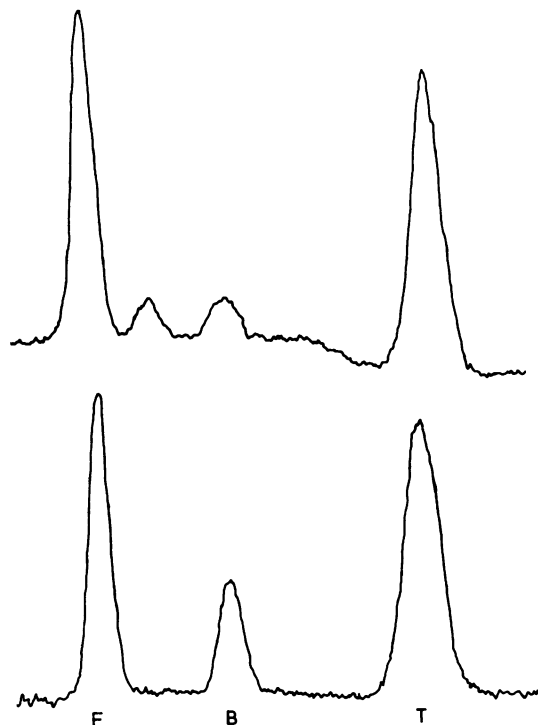


Fig 1 Typical scan given by normal male plasma (top) and steroid standards (bottom). F = 607.2 nmol/l (22 µg/100 ml) cortisol; B = 57.8 nmol/l (2.0 µg/100 ml) corticosterone; T = 17.4 nmol/l (500 ng/100 ml) testosterone.

one steroid to another, and may need to be adjusted accordingly.

EXCITATION AND EMISSION WAVELENGTHS

Choice of excitation and emission wavelengths on the spectrophotofluorimeter were made on the basis of the fluorescence spectra. For most steroids the optimal settings were 470 nm of excitation and 520 nm of emission. Spectra for cortisol, corticosterone, and testosterone were virtually identical, but were distinctive for some other steroids.

DOSE RESPONSE CURVES

There was a linear relation between the amount of steroid and the area under the curve of the peak of the scan trace. Examples of these with standard solutions of cortisol, corticosterone, and testosterone are shown in figs 2-4. The range of steroid concentrations used covered those found in plasma.

SENSITIVITY

It is possible to distinguish with certainty an area of 0.5

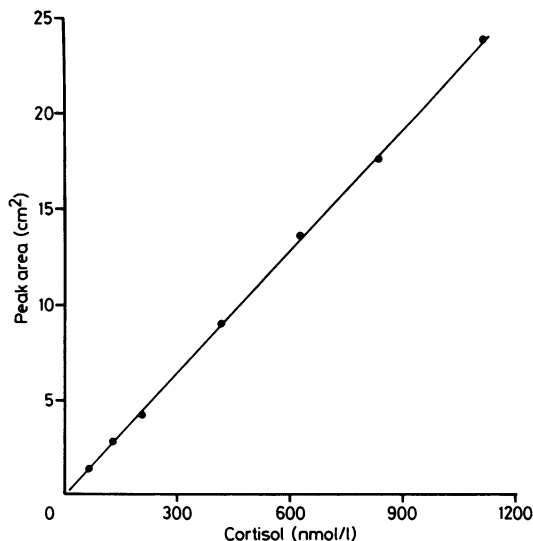


Fig 2 Dose-response curve for cortisol.

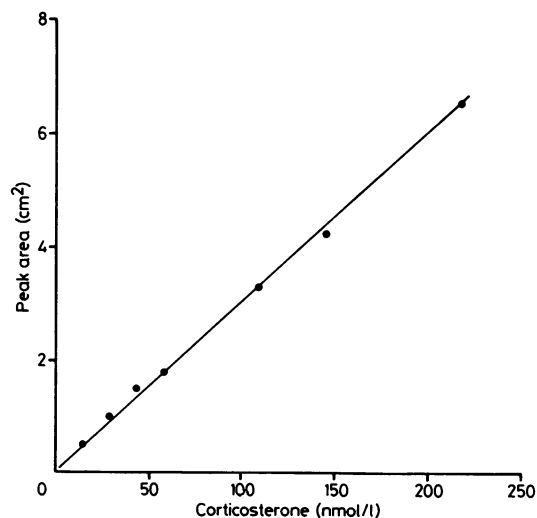


Fig 3 Dose-response curve for corticosterone.

cm² above the baseline on the scan traces. For cortisol the lower limit of detection is 16.5 nmol/l. For corticosterone it is 2.9 nmol/l, and for testosterone 0.35 nmol/l. The sensitivity of the spectrophotofluorimeter can be adjusted so that the amount of steroid/cm² scan trace is suitable for the sample under test.

Results

ACCURACY

The ability of the method to measure true values was

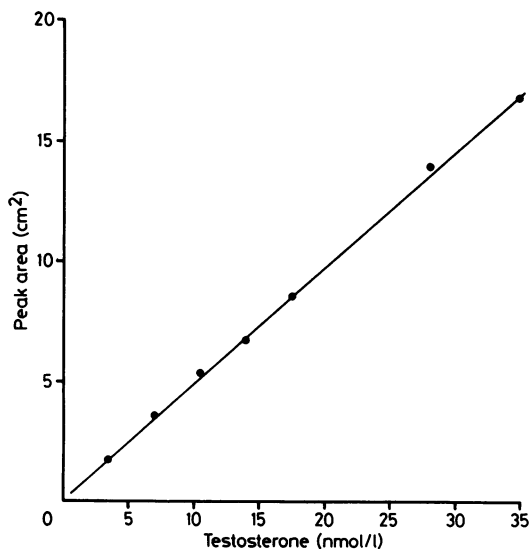


Fig 4 Dose-response curve for testosterone.

assessed by recovery experiments in which known amounts of steroid were added to the plasma before assay.

PRECISION

The precision of the method (P) was assessed both for duplicates assayed together and for replicates measured on different days. It is expressed in terms defined by Snedecor⁴ as:

$$P = \sqrt{\frac{\sum(d^2)}{2N}}$$

where $\Sigma(d^2)$ = the sum of the squared difference between duplicates or replicate estimations, and N = number of paired estimations.

The results obtained by the method described here were compared with other methods in general use, by further estimations on the same plasma sample. Values for plasma cortisol were compared with the values obtained by Mattingly's fluorimetric method for 11-OHCS,² which measures both cortisol and corticosterone, and values for plasma testosterone with an RIA method (Nordiclab kit, available from Nordiclab, Oulunsalo, Finland)

CORTISOL

The mean recovery of 8.28 nmol (0.3 µg) of cortisol added to 2 ml of plasma was 92.3% (± 6.3 (SD)), with a range of 81.7–98.1% on five estimations. The precision over our normal morning range of plasma

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cortisol concentration of 180–674 nmol/l was ± 17.7 nmol/l for 15 duplicates estimated together, and ± 33.6 nmol/l for 10 replicates estimated on different days. There was good correlation with the results obtained on the same sample using Mattingly's fluorimetric method for 11-hydroxycorticosteroids.² Of 26 plasma samples with concentrations within the normal range the coefficient of correlation was 0.87.

CORTICOSTERONE

The mean recovery of 0.58 nmol (20 ng) of corticosterone added to 2 ml of plasma was 104.7% ± 5.98 (SD), with a range of 96.9–110.5% on eight estimations. The precision over our normal morning range of plasma corticosterone concentrations of 7.5–43.9 nmol/l was ± 0.46 nmol/l for 12 duplicates measured together, and ± 0.95 nmol/l for 10 replicates measured on different days.

TESTOSTERONE

The mean recovery of 0.22 nmol (6.4 ng) of testosterone added to 2 ml of female plasma was 94.9% ± 5.9 (SD), with a range of 84–102.9% on nine estimations. The precision over our range of normal plasma testosterone concentrations in women of 0.6–3.6 nmol/l was ± 0.13 (SD) nmol/l for six duplicates measured together, and ± 0.21 nmol/l for six replicates measured on different days.

In the plasma of male patients, over our normal range of 11.7–40.1 nmol/l, the precision was ± 0.54 nmol/l for nine duplicates where the pair were measured together, and ± 1.21 nmol/l for six replicates measured on different days.

The results of 28 estimations were compared with those obtained on the same sample by the RIA Nordiclab method. The correlation coefficient was 0.88, which is satisfactory considering that this RIA method also measures small amounts of other steroids.

NORMAL VALUES

In 45 normal subjects the range of morning plasma cortisol concentrations was 180–674 nmol/l, with a mean of 365 ± 127 (SD) nmol/l. For corticosterone, the range was 7.5–43.9 nmol/l, with a mean of 21.7 ± 8.1 (SD) nmol/l. For testosterone, subjects were divided according to sex. In 23 men the concentrations ranged from 11.7–40.1 nmol/l, with a mean of 23.8 ± 9.4 (SD) nmol/l. In 22 women the range was from 0.6–3.6 nmol/l, with a mean of 1.73 ± 0.7 (SD) nmol/l.

ABNORMAL VALUES

The method was also applied to plasma from patients expected to have concentrations outside the normal range. For example, plasma from a 19 year old girl

with untreated Addison's disease showed a cortisol concentration of only 41.4 nmol/l with no detectable corticosterone; in a 65 year old man with a bronchial carcinoma and the ectopic ACTH syndrome the cortisol and corticosterone concentrations were grossly raised to 3920 nmol/l and 702 nmol/l, respectively.

In 10 untreated hypogonadal men the plasma testosterone concentration ranged from undetectable to 6.9 nmol/l. After an oral dose of 40 mg of testosterone undecanoate (Restandol-Organon) it rose to a peak between two and four hours after ingestion. This maximal concentration varied between 6.0 and 27.7 nmol/l with a mean of 17.9 nmol/l.

Discussion

The major advantages of the method described are its high specificity, its ability to detect unknown substances, and the rapidity with which results can be obtained. The criteria used to establish identity are the chromatographic behaviour of the individual steroids in different running solvents, their fluorescence spectra, and the conditions under which the fluorophores are produced. The non-production of a fluorophore in solution does not necessarily preclude its development on the TLC plate. Thus testosterone, unlike cortisol and corticosterone, gives no fluorescence when reacted with ethanolic sulphuric acid in solution at room temperature, but produces noticeable fluorescence when heated after development on a TLC plate.

The high specificity of this technique also avoids inaccuracies due to the cross-reactivity which occurs in most competitive protein binding (CPB) and RIA methods. This can be both substantial and variable. For example, prednisolone is a major source of interference in RIA assays for serum cortisol, but this cross-reactivity may vary between 22 and 60%, depending on the method used.⁵ Prednisolone and several other steroids such as progesterone and 11-deoxycortisol interfere with CPB assays. Under most circumstances these interferences are unimportant in view of the usually higher concentration of circulating cortisol. This is not the case in patients taking prednisone or prednisolone, however, where misleading high results will conceal any underlying adrenocortical deficiency. Similarly, the interference produced by high progesterone activities in pregnancy, or of 11-deoxycortisol in the metyrapone stimulation test, will give erroneous results if CPB methods are used.

The Nordiclub (1982) RIA method for the estimation of testosterone, with which our results are compared, is also subject to inaccuracy. Both 5 α -dihydrotestosterone and 5 β -dihydrotestosterone give significant amounts of cross-reaction (30% and 25%, respectively), while some other androgens cross-react less strongly. Such unwanted reactivity can be iden-

tified and avoided by using the method described here.

Any new method, whatever its advantages, must also have acceptable values for accuracy and precision, its sensitivity must be adequate for the samples under test, and the results obtained should compare with those using other established techniques. This method satisfies these criteria on all counts. The accuracy and precision are comparable with those of methods in general use, and the sensitivity is such that concentrations below the normal ranges can be measured.

The agreement of results obtained on identical samples by this and another established technique were excellent. In the case of cortisol, in 26 plasma samples assayed by the present method, and for 11-OHCS by the Mattingly method, the coefficient of correlation was 0.87. Because the latter method measures both cortisol and corticosterone, this value, slightly below unity, reflects a good agreement between the two methods. For testosterone, comparison was made with an established RIA method in general use (Nordiclub). Here again, the agreement was good with a coefficient of correlation of 0.88 on 28 plasma samples. For corticosterone, where we had no access to an alternative method of assay, it was impossible to make a direct comparison, but from data on precision, accuracy, and recovery there is no reason to believe that it is any less satisfactory than those for cortisol and testosterone. Finally, the normal values obtained by this method for all three steroids are in good agreement with those of other workers (tables 2-5).

Table 2 Morning plasma cortisol concentrations in normal adults (nmol/l)

Reference	Method	No	Mean (range)
Newsome <i>et al</i> 1972 ⁶	CPB	11	339 (248-428)
West <i>et al</i> 1973 ⁷	RIA	30	381 (110-662)
Gore and Lester 1975 ⁸	CPB	54	395 (163-627)
Matsumura 1980 ⁹	RIA	33	436 (339-533)
Urhausen and Kinderman 1987 ¹⁰	RIA	8	337 (190-486)
Present study		45	365 (180-674)

Cortisol nmol/l \times 0.0362 = μ g/100 ml.

Table 3 Morning plasma corticosterone concentrations in normal adults (nmol/l)

Reference	Method	No	Mean (range)
West <i>et al</i> 1973 ⁷	RIA	30	15.3 (2.3-31.5)
Matsumura 1980 ⁹	RIA	33	24.0 (7.8-40.2)
Holsboer <i>et al</i> 1982 ¹¹	RIA	6	14.7 (7.2-24.3)
Wilens <i>et al</i> 1984 ¹²	RIA	6	11.6 (8.7-14.7)
Oka <i>et al</i> 1987 ¹³	HPLC	20	11.3 (6.6-15.9)
Present study		45	21.7 (7.5-43.9)

Corticosterone nmol/l \times 0.0346 = μ g/100 ml.

Table 4 Plasma testosterone concentrations in normal men (nmol/l)

Reference	Method	No	Mean (range)
Ahmed 1973 ¹⁴	CPB	12	14.9 (9.7-20.8)
Williams <i>et al</i> 1974 ¹⁵	RIA	16	27.2 (13.9-41.6)
Vermeulen 1976 ¹⁶	RIA	31	20.1 (6.2-34.0)
Bassett 1980 ¹⁷	RIA	10	22.0 (14.0-31.0)
Shieh <i>et al</i> 1987 ¹⁸	EIA	19	32.7 (15.3-69.4)
Present study		23	23.8 (11.7-40.1)

Testosterone nmol/l \times 28.8 = ng/100 ml.

Table 5 Plasma testosterone concentrations in normal women (nmol/l)

Reference	Method	No	Mean (range)
Ahmed 1973 ¹⁴	CPB	12	1.60 (1.0-2.4)
Williams <i>et al</i> 1974 ¹⁵	RIA	50	2.81 (1.4-3.8)
Vermeulen 1976 ¹⁶	RIA	41	1.18 (0.7-1.6)
Bassett 1980 ¹⁷	RIA	10	2.39 (1.9-3.0)
Carter <i>et al</i> 1983 ¹⁹	RIA	32	1.94 (1.0-3.5)
Present study		22	1.73 (0.6-3.6)

Testosterone nmol/l \times 28.8 = ng/100 ml.

This method has proved extremely useful in clinical practice, particularly in confirming the diagnosis of pituitary and adrenal failure in acute medical admissions. It has also been invaluable in determining the degree of absorption of oral testosterone undecanoate in the treatment of hypogonadal men. With most other assays it is only economically viable to work with large batches of specimens, whereas the method described here is cheap and quick enough for single specimens to be processed in a working day. The method also avoids the disadvantages inherent in working with radioactive materials. In particular, it avoids inaccuracies due to cross-reactivity with other naturally occurring steroids, or interference by synthetic compounds such as prednisolone.

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References

- 1 Hamman BL, Martin MM. Direct fluorescence quantitation on thin-layer chromatograms. *J Lab Clin Med* 1969;73:1042-8.
- 2 Mattingly D. A simple fluorimetric method for the estimation of free 11-hydroxycorticosteroids in human plasma. *J Clin Pathol* 1962;15:374-9.

- 3 Ismail AAA, Lovè DN, McKinney RWJ. Elimination of plasma lipids from steroid hormone extracts by differential solubility in water. *Steroids* 1972;19:689-700.
- 4 Snedecor GW. Queries. *Biometrics* 1952;8:85-8.
- 5 Moore A, Aitken R, Burke C, *et al*. Cortisol assays: guidelines for the provision of a clinical biochemistry service. *Ann Clin Biochem* 1985;22:435-54.
- 6 Newsome HH, Clements AS, Borum EH. The simultaneous assay of cortisol, corticosterone, 11-deoxycortisol and cortisone in human plasma. *J Clin Endocrinol* 1972;34:473-83.
- 7 West CD, Mahajan DK, Chavré VJ, Nabors CJ, Tyler FH. Simultaneous measurement of multiple plasma steroids by radioimmunoassay demonstrating episodic secretion. *J Clin Endocrinol Metab* 1973;36:1230-6.
- 8 Gore M, Lester E. Comparison of a fluorimetric method and a competitive protein binding assay kit for the determination of plasma hydroxycorticosteroids. *Ann Clin Biochem* 1975;12:160-2.
- 9 Matsumura S. The effects of ACTH, insulin hypoglycaemia, dexamethasone and metyrapone on corticosterone secretion in healthy subjects: A comparison with cortisol secretion. *Folia Endocrinologica Japonica* 1980;56:129-38.
- 10 Urhausen A, Kinderman W. Behaviour of testosterone, sex hormone binding globulin (SHBG) and cortisol before and after a Triathlon competition. *Int J Sports Med* 1987;8:305-8.
- 11 Holsboer F, Winter K, Dorr HG, Sipell WG. Dexamethasone suppression test in female patients with endogenous depression: determinations of plasma corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, cortisol and cortisone. *Psychoneuroendocrinol* 1982;7:329-38.
- 12 Wilens TE, Ritchie JC, Carroll BJ. Comparison of plasma cortisol and corticosterone in the dexamethasone suppression test for melancholia. *Psychoneuroendocrinol* 1984;9:45-55.
- 13 Oka K, Noguchi M, Kitamura T, Shima S. Liquid chromatography and radioimmunoassay compared for determination of cortisol and corticosterone in plasma after a dexamethasone suppression test. *Clin Chem* 1987;33:1639-42.
- 14 Ahmed J. A method for the determination of plasma testosterone by competitive protein-binding after chromatography on sephadex LH-20. *Clin Chim Acta* 1973;43:371-8.
- 15 Williams BM, Horth CE, Palmer RF. The measurement of testosterone in plasma. *Clin Endocrinol* 1974;3:397-410.
- 16 Vermeulen A. Testosterone and 5 α androstan-17 β -ol-3-one (DHT) levels in man. *Acta Endocrinol* 1976;83:651-64.
- 17 Bassett RM. A simple chromatographic method for the radioimmunoassay of four androgenic steroids. *Med Lab Sci* 1980;37:31-8.
- 18 Shieh CC, Chang SC, Tzeng CR, Huang JJ, Nir WJ, Hong CY. Measurement of testosterone in seminal plasma, saliva and serum by solid-phase enzymeimmunoassay. *Andrologia* 1987;19:614-9.
- 19 Carter GD, Holland SM, Alagband-Zadeh J, Rayman G, Dorrington-Ward P, Wise PH. Investigation of hirsutism: testosterone is not enough. *Ann Clin Biochem* 1983;20:262-3.

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