Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 551–555. Pergamon Press. Printed in Great Britain

A SIMPLE RADIOIMMUNOASSAY FOR PLASMA TESTOSTERONE PLUS 5α-DIHYDROTESTOSTERONE

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(*Received* 3 *January* 1974)

SUMMARY

This report describes a simple and rapid radioimmunoassay for testosterone, including 5α-dihydrotestosterone, in peripheral plasma using an antiserum raised against testosterone-3-carboxymethoxime coupled to thyroglobulin. A dichloromethane extract of the plasma is assayed directly without a chromatographic step. The accuracy and precision of the method is satisfactory. The minimum amount of hormone per sample which can be measured with adequate reliability is 50 pg. The plasma testosterone levels for normal males and normal females are 725 ± 254 (S.D.) and 67·4 ± 23·7 (S.D.) ng/100 ml respectively. One technician can assay 200–250 samples in five working days. The method is suitable for routine clinical use and research purposes.

INTRODUCTION

Although a number of methods has been described in the past few years for the measurement of testosterone by radioimmunoassay [1–8] most of these methods employ a chromatographic step—paper, thin layer or column chromatography to achieve specificity. This report describes a simple and rapid radioimmunoassay for the measurement of plasma testosterone. A dichloromethane extract of the plasma is assayed directly with an antiserum raised against testosterone-3-carboxymethoxime coupled to thyroglobulin. This provides a rapid and simple assay with reasonable specificity acceptable to routine clinical use and also for research purposes.

EXPERIMENTAL

Antiserum

The antigen used was testosterone-3-carboxymethoxime coupled to porcine thyroglobulin by the method of Chopra *et al.* [9]. 100 μg of this conjugate was dissolved in 1·0 ml 0·15 mol/l saline, mixed with 5 mg dried tubercle bacilli and emulsified with 1 ml of complete Freund's adjuvant. The method of initial administration was similar to that described by Vaitukaitis *et al.* [10]. The emulsified antigen was given intradermally to rabbits at 20 sites along the back. Three and four months later two booster doses of 1 mg of the conjugate in 1 ml 0·15 mol/l saline emulsified with 1 ml of complete Freund's adjuvant, were given subcutaneously. Five months after the injection schedule was commenced, the final antiserum was obtained. The antiserum was diluted in 1:100 with 0·05 mol/l borate buffer, pH 8·0 and 0·1 ml aliquots were stored at −20°C until required. Before each assay an aliquot of the antiserum was diluted with borate buffer to a dilution of 1:1500.

Reagents

Dichloromethane was purified by passage through a silica gel column and stored at −20°C before use [11].

Borate buffer 0·05 mol/l, pH 8 was used throughout the assay and contained 0·06% BSA and 0·05% human gamma globulin, the former to minimize adsorption of steroid to the walls of the assay tubes and the latter to increase the mass of the final precipitate.

Testosterone standards (K & K Laboratories, Plain View, New York) were prepared weekly in borate buffer and stored at +4°C in suitable dilutions from a stock solution of 100 ng/ml in ethanol.

[1,2-^{3}H]-testosterone (56 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England, and purified by thin layer chromatography in the system, benzene–ethylacetate, 2:1 (v/v). The purified steroid was stored in ethanol at +4°C.

Plasma samples

Blood was collected into heparinized tubes and the cells separated from the plasma within 1 h. The plasma was stored at −20°C until used.

Procedure

100–500 μl of plasma (usually 100 μl of human male plasma or 500 μl of female plasma) is used. In order to monitor losses 1000 d.p.m. [1-2^{3}H]-testosterone (2·3

pg) is added to each sample prior to extraction. Samples are extracted with 20 ml dichloromethane and the organic phase washed once with 2 ml distilled water and then dried in a stream of air at 35°C. Two ml of borate buffer is then added to each dried extract and mixed on a Vortex mixer. Two 0·5 ml aliquots (Oxford pipette) are transferred to 10 ml disposable polystyrene assay tubes and another 0·5 ml aliquot to a counting vial to determine recovery.

In order to minimize plasma and reagent blank material, an equivalent amount of plasma, pretreated with charcoal (50 mg/ml) to remove endogenous steroids ("stripped plasma") is extracted in the same way as the plasma samples and 0·5 ml of the final extract added to each standard curve tube.

Duplicate 50 μl aliquots of standard testosterone solutions are added to each of the tubes of the standard curve to give final masses of 50, 100, 150, 200, 300 and 400 pg of testosterone. A similar volume of buffer is added to 2 control tubes, the sample tubes and 2 assay tubes that are subsequently used to estimate the total amount of tracer added to the assay system ("saline" tubes). A sufficient volume of the antiserum is mixed with the tracer such that 50 μl of the antiserum (1:1500 dilution) contains 55,000 d.p.m. [1,2-^{3}H]-testosterone (128 pg). After mixing, 50 μl of tracer antiserum is added to each of the standard, control, saline and assay tubes to give a final dilution of 1:18,000 and an incubation volume of 0·6 ml. The tubes are then sealed with Parafilm and incubated overnight at +4°C.

Separation of free and bound testosterone is achieved by addition of 0·6 ml of saturated ammonium sulphate to all except the saline tubes to which 0·6 ml 0·15 mol/l saline is added. The contents of the tubes are mixed immediately, allowed to stand for 10 min at +4°C and then centrifuged at 1450 g for 20 min. Half millilitre aliquots are removed from each supernatant and transferred to counting vials containing 5 ml of toluene based phosphor (0·4% PPO and 0·004% dimethyl POPOP) and the free radioactivity measured in a Packard liquid scintillation counter such that the counting error is less than ±2·5%.

Standard curves are constructed with the per cent bound steroid plotted as a function of the mass of testosterone. The concentration of testosterone in ng/100 ml is derived from the following equation.

$$\underset{\text{(from curve)}}{\text{pg/sample}} \times \frac{100}{\text{recovery }\%} \times \frac{1}{\text{volume of plasma}} \times \frac{1}{10}$$

RESULTS

Recovery

The mean recovery of [1,2-^{3}H]-testosterone from plasma was 91·9 ± 4·8% (n = 42).

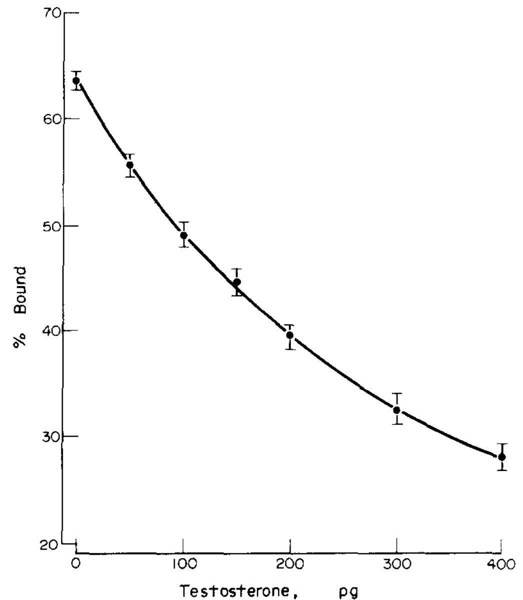

Fig. 1. Testosterone standard curve. 1:18,000 dilution of antiserum, 128 pg [1-2-^{3}H]-testosterone/0·6 ml reaction volume. The points represent the means and the bars indicate one S.E. of the mean of 8 standard curves derived from different assays.

Standard curves

A typical standard curve is shown in Fig. 1. The variability of the curve is also shown by the S.E.s of the mean of 8 standard curves derived from different assays.

Method blank

The characteristics of this parameter were evaluated by assaying increasing vol. (25, 125 and 250 μl per assay tube) of charcoal stripped plasma against a standard curve prepared in the absence of plasma extract. These results are shown in Fig. 2. It may be seen that the blank arising from charcoal stripped plasma is

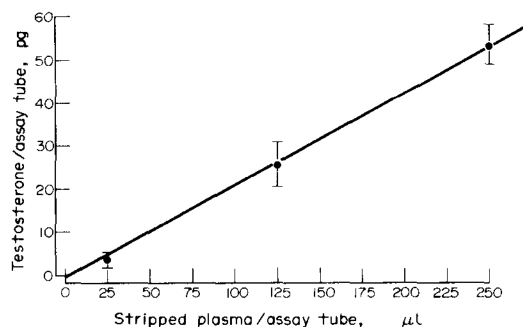

Fig. 2. Values of testosterone measured against increasing volumes of stripped plasma. (Correlation coefficient = 0·98, $y = 0{\cdot}22x - 1{\cdot}73$).

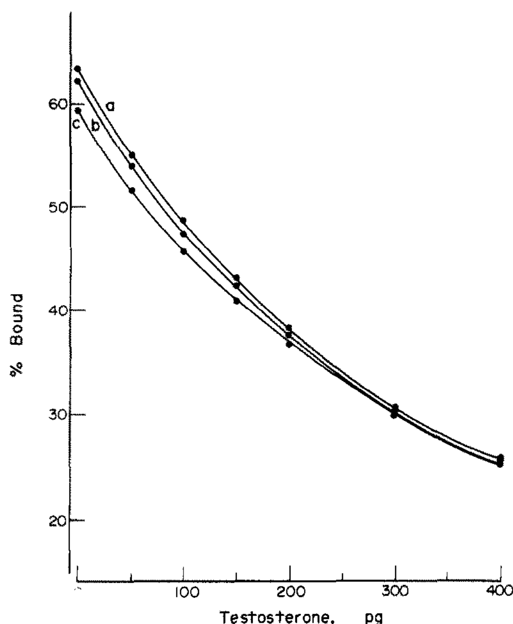

Fig. 3. Testosterone standard curves. 1:18,000 dilution of antiserum, 128 pg [1-2-^{3}H]-testosterone/0·6 ml reaction volume with no charcoal stripped plasma (curve a), the equivalent of 25 μl (curve b for male samples) and 125 μl stripped plasma per standard curve tube (curve c for female samples).

volume dependent, being 3·7 ± 1·9 pg and 25·9 ± 6·3 pg for vol. of plasma (25 and 125 μl per assay tube) normally used for the assay of male and female plasma samples respectively. In view of these findings standard curves used for the assay of plasma samples are prepared in the presence of an extract of 25 μl of stripped plasma for male samples and 125 μl for female samples (Fig. 3). The non specific blank of the method is 0·2 ± 1·9 pg per assay tube which is not significantly different from zero.

Specificity

The antiserum was examined for its cross reactivity with a number of different steroids. The relative activities of these steroids, as defined, are detailed in Table 1. The only naturally occurring steroid which showed significant cross reactivity was 5α-dihydrotestosterone; the synthetic steroid mesterolone (17β-hydroxy-1α methyl-androstan-3-one) also showed significant cross reactivity. To investigate further the interference of 5α-dihydrotestosterone quantitatively, 1000 pg of 5α-dihydrotestosterone was added to 0·1 ml stripped plasma and processed through the method, it was measured as though it was equivalent to 777 ± 17 pg of testosterone (n = 5).

Table 1. Relative activity* of selected steroids in the radioimmunoassay system

Steroid	Relative activity*
Testosterone	1·000
5α-dihydrotestosterone	0·644
Androstenedione	0·024
5-androstene-3β-17β-diol	0·0095
Progesterone	0·0025
Dehydroepiandrosterone	0·0014
Oestradiol-17β	0·0001
Oestrone	<0·00001
Cortisol	0·00002
17β-hydroxy 17α-methyl-4-androstene-3 one (methyl testosterone)	0·018
17β-hydroxy-1α-methyl-androstan-3 one (Mesterolone)	0·586

* Relative activity

$$= \frac{\text{Mass of testosterone at 50\% tracer displacement}}{\text{Mass of steroid at 50\% tracer displacement}}.$$

Precision

This was assessed by the measurement of replicate samples in the same and different assays. The intra-assay precision measured as the coefficient of variation of 28 replicates of male pooled sample was 10·0% (mean 607·1 ± 60·8 ng/100 ml) and of 13 replicates of a female pooled sample was 8·0% (mean 48·2 ± 3·8 ng/100 ml). The inter-assay precision for the same male pooled plasma sample included in 40 different assays was 10·7% (mean 579·0 ± 61·9 ng/100 ml).

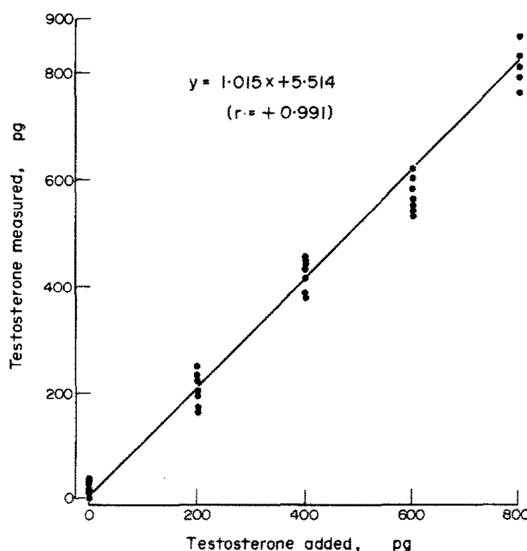

Fig. 4. Accuracy of the method. Testosterone added to charcoal stripped plasma.

Accuracy

The accuracy of the method was determined by adding 200, 400, 600 and 800 pg testosterone to charcoal stripped plasma. The plot of testosterone measured against the testosterone added is shown in Fig. 4.

Sensitivity

The smallest quantity of testosterone that can be precisely distinguished from zero is 10 pg per tube; accounting for handling losses, multiple aliquots for radioimmunoassay and an aliquot for determining recovery, the minimal amount of testosterone in a plasma sample that can be measured with adequate reliability is of the order of 50 pg.

Normal values

Human. The level of testosterone in peripheral plasma was measured in 58 normal males aged between 20–59 and 20 normal females aged between 20–45; the respective values for the groups were $724 \cdot 8 \pm 253 \cdot 8$ and $67 \cdot 4 \pm 23 \cdot 7$ ng/100 ml respectively.

Rats. The level of peripheral plasma testosterone of Sprague–Dawley rats (aged 56–63 days) was $370 \cdot 1 \pm 267 \cdot 4$ ng/100 ml ($n = 7$). The level of plasma testosterone 4 weeks after castration fell to $52 \cdot 1 \pm 21 \cdot 1$ ng/100 ml ($n = 5$) and 1 week after castration and adrenalectomy plasma testosterone was undetectable, i.e. less than 8 ng/100 ml ($n = 5$) in all animals.

DISCUSSION

The radioimmunoassay described in this paper has been established using an antiserum that has a high degree of specificity for testosterone. Comment should perhaps be made about the use of porcine thyroglobulin as the protein carrier. This protein was used because of the report by Skowsky and Fisher [12]. These authors compared the antibody responses achieved using lysine vasopressin coupled to thyroglobulin with those when this small peptide was coupled to bovine serum albumin; 100% of the animals immunized with a lysine vasopressin-thyroglobulin conjugate showed satisfactory antibody responses compared with zero responses to the lysine vasopressin BSA conjugates. In these laboratories steroid BSA conjugates had been notably unsuccessful in evoking useful antibody response; furthermore, a series of steroid thyroglobulin conjugates have enabled antibodies to be produced for a variety of steroids. We would agree with Odell *et al.* [13] that conjugation to thyroglobulin is the simplest, most productive method resulting in useful antisera not only to small non-immunogenic polypeptides, but also to steroids. There is no evidence which would suggest that conjugation with thyroglobulin enhances the specificity of the antiserum, in fact antibodies raised against cortisol and dehydroepiandrosterone coupled with thyroglobulin show substantially different profiles of specificity (Wang *et al.*, unpublished observations).

The only naturally occurring steroid which showed significant cross reaction with the antiserum is 5α-dihydrotestosterone. 1000 pg of 5α-dihydrotestosterone carried through the procedure is measured as though it is equivalent to 777 pg of testosterone. The levels of 5α-dihydrotestosterone in plasma have been determined by Ito and Horton using a competitive protein binding technique [14]. Because this present method does not involve a chromatographic step to separate 5α-dihydrotestosterone from testosterone, the overestimate of testosterone in a normal male and female plasma sample is approximately 41 and 12 ng/100 ml respectively. In males over 70% of 5α-dihydrotestosterone is derived from peripheral conversion of testerone. In females, however, most of the 5α-dihydrotestosterone is derived from conversion of androstenedione [15] and over 60% of testosterone is also derived by conversion from androstenedione [16]. Thus, in females, testosterone and 5α-dihydrotestosterone have a common predominant precursor, androstenedione. Inclusion of 5α-dihydrotestosterone in the present radioimmunoassay does not reduce the value of the method for measuring plasma testosterone for routine clinical use and research purposes. Omission of chromatography prior to assay has the advantage of greatly simplifying the procedure so that one technician can process 200–250 samples in 5 working days.

By using charcoal stripped plasma in the standard curve, a blank in the method is low or absent. The sensitivity, precision and accuracy of the method are comparable to those described for other methods. The values for normal male and female plasma are similar to those previously reported [1–8]. The plasma testosterone in adult rats is comparable to those previously reported [17, 18]. The values for castrate rats are higher than those reported by Coyotupa *et al.* [19]. However, plasma testosterone becomes undetectable after castration and adrenalectomy, which suggests that the testosterone value after castration in these animals reflects the heightened secretion of testosterone by the adrenals as previously noted by Bardin *et al.* [20].

The small volumes of plasma (100–500 μl) required for the assay is of value when multiple hormonal determinations are being undertaken on the same plasma sample, as in studies designed to investigate interrelationships of several hormones during sleep or in testing

the effects of drugs and gonadotropin on testosterone secretion. It is of special merit when plasma testosterone measurements are required in small animals such as the rat.

Acknowledgements—This work has been supported by the National Health and Medical Research Council of Australia, and was undertaken by one of the authors (CW) during her tenure as a Commonwealth Fellow from the University Department of Medicine, Hong Kong. The technical assistance of Mr. R. Harrison is gratefully acknowledged.

REFERENCES

1. Furuyama S., Mayes D. and Nugent C. A.: *Steroids* **16** (1970) 415–428.
2. Chen J. C., Zorn E. M., Halberg M. C. and Wieland R. G.: *Clin. Chem.* **17** (1971) 581–584.
3. Dufau M. L., Catt K. J., Tsuruhara T. and Ryan D.: *Clin. chem. Acta* **37** (1972) 109–116.
4. Ismail A. A. A., Niswender G. D. and Midgley A. R.: *J. clin. Endocr. Metab.* **34** (1972) 177–184.
5. Lee P. A., Jaffe R. B., Midgley A. R., Kohen F. and Niswender G. D.: *J. clin. Endocr. Metab.* **35** (1972) 636–641.
6. Collins W. P., Mansfield M. D., Alladina N. S. and Sommerville I. F.: *J. steroid Biochem.* **3** (1972) 333–348.
7. Pirke K. M. *Acta Endocr.* **74** (1973) 168–176.
8. Kinouchi T., Pages L. and Horton R.: *J. Lab. clin. Med.* **82** (1973) 309–316.
9. Chopra I. J., Nelson J. C., Solomon D. H. and Beall G. N.: *J. clin. Endocr. Metab.* **32** (1971) 299–308.
10. Vaitukaitis J., Robbins J. B., Nieschlag E. and Ross G. T.: *J. clin. Endocr. Metab.* **33** (1971) 988–991.
11. Hudson B., Coghlan J., Dulmanis A., Wintour M. and Ekkel I.: *Austr. J. exp. Biol. med. Sci.* **41** (1963) 235–246.
12. Skowsky W. R. and Fisher D. A.: *J. Lab. clin. Med.* **80** (1972) 134–144.
13. Odell W., Skowsky R., Abraham G., Hescox M., Fisher D. and Grover P. K.: *Biol. Reprod.* **6** (1972) 427–442.
14. Ito I. and Horton R.: *J. clin. Endocr. Metab.* **31** (1970) 362–368.
15. Ito I. and Horton R.: *J. clin. Invest.* **50** (1971) 1621–1627.
16. Horton R. and Tait J. F.: *J. clin. Invest.* **45** (1966) 301–313.
17. Bartke A., Steele R. E., Musto N. and Caldwell B. V.: *Endocrinology* **92** (1973) 1223–1228.
18. de Jong F. H., Hey A. E. and van der Molen H. J.: *J. Endocr.* **57** (1973) 277–284.
19. Coyotupa J., Parlow A. F. and Kovacic N.: *Endocrinology* **92** (1973) 1579–1581.
20. Bardin C. W. and Peterson R. E.: *Endocrinology* **80** (1967) 38–44.