Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 529–532. Pergamon Press. Printed in Great Britain

SHORT COMMUNICATION

RADIOIMMUNOASSAY OF PLASMA AETIOCHOLANOLONE

E. YOUSSEFNEJADIAN, S. S. VIRDEE and I. F. SOMMERVILLE

Department of Biochemical Endocrinology, Chelsea Hospital for Women, and the Institute of Obstetrics and Gynaecology, University of London, London S.W.3.

(*Received* 29 *November* 1973)

SUMMARY

A rapid procedure is described and evaluated for the determination of free aetiocholanolone (5β-androstan-3α-ol-17-one) in peripheral venous plasma (1·0 ml) of men and women. Tritiated aetiocholanolone is added as internal standard; the plasma extracted with diethyl ether and the extract purified on pre-coated silica gel plates before radioimmunoassay using an antiserum to aetiocholanolone-3-hemisuccinate-bovine serum albumin. The range of values (mean ± S.D.) in plasma from 40 healthy men was 40·2 ± 23·8 ng/100 ml and the corresponding value for 20 healthy women was 34·5 ± 7·23.

INTRODUCTION

During the past 15 yr there have been reports which suggest that aetiocholanolone may be a steroid of especial interest. Thus, it was the first of the 5β-3α-hydroxysteroids to be shown to induce pyrexia in human subjects [1, 2, 3]; evidence for excessive production was reported in patients with Mediterranean and other periodic fevers [4] and urinary excretion was studied extensively in attempts to predict the clinical response of patients with mammary carcinoma [5]. Aetiocholanolone glucuronoside was first determined in human peripheral plasma by gradient elution chromatography on alumina with paper chromatography and spectrophotometry [6]. Subsequently, free aetiocholanolone was determined by double isotope derivative formation with gas–liquid chromatography as the final step [7] and by a similar technique involving t.l.c. [8].

The object of the present work was to develop a radioimmunological method for the determination of ether-extractable aetiocholanolone in peripheral venous plasma and to ensure that there was adequate separation from its C5 stereosiomer androsterone (5α-androstan-3α-ol-17-one). As the details of the radioimmunological procedures employed in this department have been reported elsewhere [9–13], the report is limited to a brief description and evaluation of the method with preliminary results in healthy subjects.

MATERIAL

Solvents and reagents

Diethyl ether (peroxide free), acetone and benzene, were all Analar grade and redistilled before use. Water was glass distilled and deionized. The red dye, a diazo compound of colour index no. 21260, described as solvent red 18 was obtained from Speedry Magic Marker Ltd. The pre-coated silica-gel F-254 Merck fast running plates were obtained from Anderman and Co. Ltd.

Standards

Aetiocholanolone, dehydroepiandrosterone (3β-hydroxyandrost-5-ene-17-one), androstenedione (4-androstene-3,17-dione), androsterone (5α-androstan-3α-ol-17-one), 11-ketoandrosterone (5α-androstan-3α-ol-11,17-dione), dihydroandrosterone (5α-androstan-3α,17β-diol), 11β-hydroxyandrosterone (5α-androstan-3α,11β-diol-17-one), epiandrosterone (5α-androstan-3β-ol-11,17-dione), 11β-hydroxyaetiocholanolone (5β-androstan-3α,11β-diol-17-one), aetiocholan-3α-ol-17-one (5β-androstan-3β-ol-17-one), were obtained from MRC Steroid Reference Collection, Westfield College, London N.W.3. Aetiocholanolone hemisuccinate was obtained from Steraloids Inc., Pawling, New York. Testosterone, progesterone, 17-hydroxyprogesterone, oestrone, oestradiol, oestriol, and cholesterol were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A.

[1,2-^{3}H]-aetiocholanolone (S.A. 40–50 Ci/mmol) was obtained from New England Nuclear, 6072 Dreieichenhain bei Frankfurt/Main, Siemensstrasse 1, Germany. A working solution was prepared containing 10 mCi/ml in Benzene. 100 ml of this solution were removed, dried and re-dissolved in 10 ml of phosphate buffer. 100 μl containing approximately 20,000 d.p.m. were used in the assay.

Preparation of phosphate buffer (P.B.) and dextran-coated charcoal have previously been described [8–10].

Preparation of antigen and antiserum

50 mg of aetiocholanolone–hemisuccinate was joined to BSA using a mixed anhydried reaction according to the method of Erlanger *et al.* [14].

For immunization, 1 mg of antigen was dissolved in 0·1 ml of sterile isotonic saline and emulsified with an equal volume of Freund's complete adjuvant. This emulsion was injected, subcutaneously, intramuscularly and intradermally, at multiple sites into a rabbit. The above procedure was repeated once a week for a further 3 weeks, then once

a fortnight for the following month, and subsequently once a month. Of the two rabbits so treated, both developed antisera which was used at a final dilution of 1:2000 (v/v), 12 weeks after the first injection. Neat antiserum was diluted to 1:5 (v/v) with P.B. and divided into 1 ml aliquots. These were stored at −15°C, the final dilution was made prior to use.

METHOD

Peripheral venous blood was obtained from normal subjects by means of a disposable syringe. Plasma was removed after centrifugation from tubes containing lithium heparin. This plasma was subjected to extraction immediately, or stored at −15°C for future extraction.

Assay of aetiocholanolone was conducted along similar lines as those previously described [9–13].

Briefly, tritiated aetiocholanolone (2000 d.p.m./5 pg) was added to 1 ml of plasma. This was extracted with diethyl ether (2 × 6 ml) using a vortex mixer. The extract was then transferred to a pointed tube with a pasteur pipette, and dried under nitrogen at 40°C. The residue was dissolved in four drops of dye in ether. This dye mixture was spotted onto a silica gel plate, at intervals of 2 cm.

The plate was developed in a tank previously saturated with ether. After 1·5 h the plate was removed.

Areas between the two dye bands of the plate (Fig. 1) were eluted with ether into counting vials. The samples were dried and redissolved in 1·0 ml of acetone. An aliquot of 20% was removed (in triplicate) for assay, and the remainder subjected to liquid scintillation counting, in order to correct for experimental losses.

A standard curve (0–200 pg), and unknowns were prepared in triplicate, dried and equilibrated with antiserum (100 µl) for 30 min at room temperature. After this 100 µl of tritiated aetiocholanolone (20,000 d.p.m./50 pg) was added to each tube. The tubes were mixed and allowed to equilibrate overnight at 4°C.

The unbound steroid was removed by the addition of 1 ml of dextran-coated charcoal followed by centrifugation.

The antibody bound to steroid was decanted into a counting vial containing scintillation fluid which consisted of toluene and Triton X-100 in the ratio of 2:1 v/v and 6 g of P.P.O./l. of toluene.

CALCULATION OF RESULTS

A standard curve (0–200 pg), where the amount of tritiated aetiocholanolone bound to antibody is expressed as d.p.m., is shown in Fig. 2. The mean number of d.p.m. in the samples removed for assay was calculated, and the corresponding values in pg were read from the standard curve and corrected for aliquots taken, experimental losses and volume of plasma. The final results were expressed as ng/100 ml of plasma a desk-top computer being used to calculate the results.

Specificity

The specificity of the method depends upon the characteristics of the antiserum. Several steroids with similar chemical structures have the ability to compete for binding sites on the antibody to aetiocholanolone hemisuccinate. Table 1 shows the ability of each steroid tested to compete for

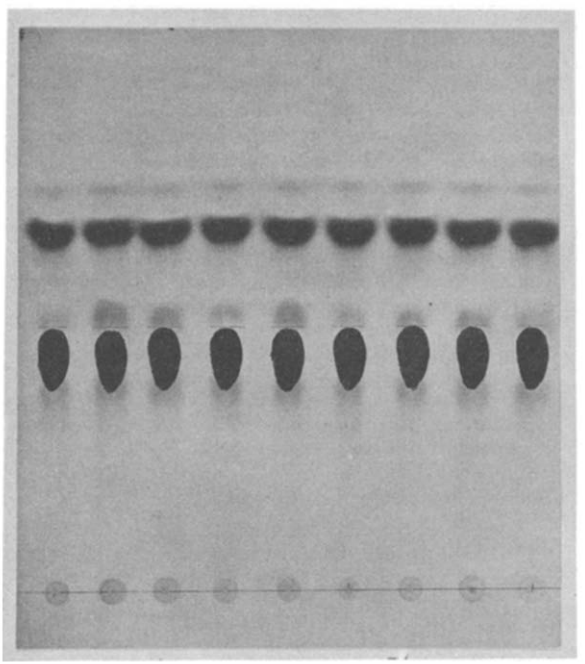

Fig. 1. The position of the area between the two bands where aetiocholanolone is eluted from the plate.

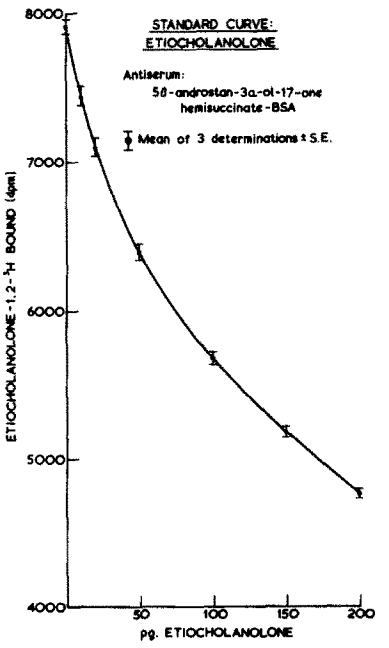

Fig. 2. Standard curve for aetiocholanolone, using an antiserum to aetiocholanolone hemisuccinate-BSA at a final dilution of 1:2000 (v/v).

Table 1.

Steroids	% Cross reaction relative to aetiocholanolone
Aetiocholanolone	100·0
Testosterone	<0·1
Dehydroepiandrosterone	<0·1
Androstenedione	<0·1
Androsterone	39·6
11-Keto androsterone	<0·1
Dihydroandrosterone	<0·1
11β-Hydroxyandrosterone	<0·1
Epiandrosterone	3·6
11-Keto aetiocholanolone	3·0
11β-Hydroxyaetiocholanolone	<0·1
Aetiocholan-3β-ol-17-one	<0·1
Progesterone	<0·1
17-Hydroprogesterone	<0·1
Oestrone	<0·1
Oestradiol	<0·1
Oestriol	<0·1
Cholesterol	<0·1

binding sites on the antibody. This was investigated by testing each steroid for its ability to displace tritiated aetiocholanolone from the antiserum. A figure for expressing the degree of cross-reactivity was derived by expressing the mass of unlabelled aetiocholanolone required to displace 50% of the label from the antiserum as a percentage of the corresponding mass of competing steroid. Androsterone had the ability to compete for binding sites as much as 39%. This necessitated the inclusion of the chromatographic step.

Method of blank

Deionized distilled water (1 ml) was extracted with ether, dried under nitrogen and, after addition of dye was spotted on the plate directly without addition of steroid. The area between the two dye bands was eluted. The results obtained are shown in Table 2.

Table 2. Method blanks

No. of determinations	Mean ± S.D. ng/100 ml	Range
20	2·39 ± 1·63	0·00–4·00

Accuracy

The accuracy of the method was established by carrying out replicate analysis on 1 ml of distilled deionized water, or plasma to which known amounts of authentic steroid had been added.

Precision

The precision of the method was assessed by carrying out replicate determinations on both male and female plasma pools. The results are reported in Table 4.

Table 3. Replicate analysis of authentic aetiocholanolone added to plasma and deionized distilled water

Amount added	No. of determinations	Amount calculated ng/100 ml	Coefficient of variation (%)
0·0 pg/1·0 ml	5	18·97	10·7
100 pg/1·0 ml	5	30·00	11·2
200 pg/1·0 ml	5	40·17	11·5
500 pg/1·0 ml	5	69·60	9·9
1000 pg/1·0 ml*	20	106·00	9·4

* Added to deionized distilled water.

Sensitivity

The lower limit of aetiocholanolone which can be accurately determined, depends on; the volume of plasma extracted, the aliquot removed for assay, the value obtained from the method of blank and the experimental error. From the data obtained, and in view of the above facts, the lower limit of detection was estimated to be 3 ng/100 ml. The values of blanks fall well below this figure.

Table 4. Replicate analysis of plasma aetiocholanolone in male and female plasma pools

	No. of determinations	ng/100 ml Mean ± S.D.	Coefficient of variation (%)
Male plasma	20	74·5 ± 9·9	13·4
Female plasma	20	54·8 ± 7·10	12·9

Results

The mean and range of values for plasma aetiocholanolone for both men and women and the percentage recoveries are given in Table 5.

Table 5. Plasma aetiocholanolone levels in healthy males and females

	No. of determinations	ng/100 ml Mean ± S.D.	Range	Recoveries (%)
Male plasma	40	40·2 ± 23·8	29–77	70
Female plasma	20	34·5 ± 7·23	27–45	75

DISCUSSION

In a similar technique for the radioimmunoassay of plasma androsterone [13] an antiserum to androsterone-17-carboxymethyl oxime was used and the percentage cross-reaction of aetiocholanolone and numerous C_{19} steroids was less than 10%. In the present technique the antiserum was less specific and the percentage cross-reaction with

androsterone was of the order of 40%. For this reason preliminary purification of the extract is essential. Initially, separation of the two C^5 stereoisomers was attempted on micro-columns of Sephadex LH20 but this was not successful whereas adequate separation was obtained using the pre-coated silica gel plates. It should be noted that the incorporation of this chromatographic step did not significantly raise the blank value which was, in fact, lower than that obtained in the technique for plasma androsterone where preliminary purification was not employed. With regard to the specificity of the method, it should be noted that values obtained in healthy human subjects are considerably lower than those obtained by previous methods, with the exception of the time-consuming but specific double isotope derivative technique [8].

REFERENCES

1. Kappas A., Hellman L., Fukushima D. K. and Gallagher T. F.: *J. clin. Endocr. Metab.* **17** (1957) 451–453.
2. Segaloff A., Bowers C. Y., Gordon D., Schlosser J. V. and Murison P. J.: *Cancer* **10** (1957) 1116–1118.
3. Kappas A., Hellman L., Fukushima D. K. and Gallagher T. F.: *J. clin. Endocr. Metab.* **18** (1958)1043–1055.
4. Bondy P. K., Cohn G. L. and Gregory P. B.: *Medicine* **44** (1965) 249–261.
5. Bulbrook R. D., Greenwood F. C. and Hayward J. L.: *Lancet* **1** (1960) 1154–1157.
6. Kellie A. E. and Smith E. R.: *Biochem. J.* **66** (1957) 490–494.
7. Kirschner M. A., Lipsett M. B. and Collins D. R.: *J. clin Invest.* **44** (1965) 657–665.
8. George J. M., Wolff S. M., Diller E. and Bartter F. C.: *J. clin. Invest.* **48** (1969) 558–563.
9. Emment Y., Collins W. P. and Sommerville I. F.: *Acta Endocr., Copenh.* **69** (1972) 567–582.
10. Collins W. P., Mansfield M. D., Alladina N. S. and Sommerville I. F.: *J. steroid Biochem.* **3** (1972) 333–348.
11. Youssefnejadian E., Florensa E., Collins W. P. and Sommerville I. F.: *J. steroid Biochem.* **3** (1972) 893–901.
12. Youssefnejadian E., Florensa E., Collins W. P. and Sommerville I. F.: *Steroids* **20** (1972) 773–788.
13. Youssefnejadian E., Collins W. P. and Sommerville I. F.: *Steroids* **22** (1973) 63–72.
14. Erlanger B. F., Berek F., Bieser S. M. and Lieberman S.: *J. biol. Chem.* **228** (1957) 713–727.