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SHORT COMMUNICATION

THE MEASUREMENT OF 5-ANDROSTENE-3β, 17β-DIOL IN PLASMA BY RADIOIMMUNOASSAY

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SUMMARY

A sensitive and specific radioimmunoassay for the measurement of 5-androstene-3β, 17β-diol (androstenediol) in plasma has been developed using an antiserum against androstenediol-7-carboxymethyloxime conjugated to bovine serum albumin. Plasma samples were extracted in diethylether and chromatographed on Sephadex LH-20 prior to assay. Plasma androstenediol levels were measured in 113 normal women between the ages of 30 and 70 yr. The mean values ($\pm$SD) for women in the age ranges 30–50 and 51–70 were 552 $\pm$ 223 pg/ml and 401 $\pm$ 229 pg/ml respectively.

Plasma levels of 5-androstene-3β, 17β-diol (androstenediol) have previously been measured by competitive protein binding assays using sex hormone binding globulin [1–3] and by radioimmunoassay utilizing antisera raised against dehydroepiandrosterone coupled through the C-17 position to bovine serum albumin [4]. Since these binding proteins were not specific for androstenediol, extensive purification of sample extracts was required before assay. Radioimmunoassays using antisera generated against androstenediol coupled through the C-16 position to the carrier proteins have recently been published [5, 6]. These antisera cross-reacted significantly with other 5-ene-3β-OH and 17β-OH steroids, however, and thin-layer or paper chromatography was required for a specific assay.

This communication presents a simple and specific assay using an antiserum against androstenediol coupled through the C-7 position to bovine serum albumin and investigates the range of plasma levels in a normal female population.

The assay buffer was prepared by dissolving 0.1% gelatin and 0.1% sodium azide in 0.16 M phosphate buffered saline (pH 7.0). The dextran-coated charcoal (DCC) suspension used to separate the antibody-bound steroid from the free fraction, was prepared by adding 0.5% of Norit A (Sigma Chemical Co., St. Louis, MO) and 0.05% of Dextran T70 (Pharmacia Fine Chemicals, Uppsala) to assay buffer. Androstenediol and steroids for cross reactivity studies were obtained from Steraloids Ltd., Croydon, England and 1,2[^{3}H]-androstenediol (SA 58 Ci/mmol) from New England Nuclear Corporation, Boston, MA). Sephadex LH-20 was obtained from Pharmacia and Woelm neutral alumina for column chromatography from I.C.N. Pharmaceuticals, GmbH and Co., 3440 Eschwege, West Germany.

The immunogen was prepared by coupling androstenediol-7-o-carboxymethyloxime, synthesised as previously described [7], to bovine serum albumin by the mixed anhydride reaction [8]. The addition of tracer amounts of radiolabelled steroid at the start of the synthesis facilitated the measurement of the molar incorporation of the steroid into the protein. This was determined to be 33. Four New Zealand white rabbits were immunised using a similar schedule to that previously described [9] and bled out after 27 weeks, the titres being 1:30,000(R1), 1:17,000(R2), 1:20,000(R3 and R4).

Cross reactions [10] of the antisera with a range of steroids were all low except for 5α-androstane-3β-17β-diol which is not easily separated chromatographically from androstenediol. The antiserum (R1) which cross-reacted least with this steroid was chosen for the assay and its specificity is shown in Table 1. The final dilution of the antiserum which maximally displaced 600 pg of androstenediol [11] was 1:13,000. This dilution, which gave an initial binding of 70%, was used in the assay.

For the radioimmunoassay diethylether extracts were prepared from plasma (1.5–2.0 ml) to which 1500 d.p.m. of 1,2[^{3}H]-androstenediol had been added to monitor losses. After solvent evaporation, extracts were chromatographed on 12 × 1 cm LH-20 columns equilibrated and developed in benzene–methanol (85:15, V/V). The first 7 ml of eluate were discarded and the next 3.5 ml, the androstenediol fraction, collected and the solvent evaporated. The dried fraction was dissolved in 700 μl of assay buffer and a portion (200 μl) counted to estimate the recovery of labelled tracer. Duplicate aliquots (200 μl) were transferred to assay tubes and a range of androstenediol standards in buffer (200 μl) containing 0–600 pg per tube set up. All tubes were incubated at 37°C for 20 min with 150 μl of the antiserum premixed with 20,000 d.p.m. of 1,2[^{3}H]-androstenediol and cooled to 0°C. DCC (300 μl) was added, with mixing and after 40 min at 0° the tubes were centrifuged at 2000 *g* for 15 min in a refrigerated centrifuge. A portion (500 μl) of the supernatant was counted and results calculated by curve fitting to a third order equation on a Wang 2200 mini computer.

To determine whether the assay could be applied directly to crude ether extracts, plasma samples from nine normal females were analysed before and after chromatography on the Sephadex LH-20 system described. The mean androstenediol level ($\pm$SD) obtained without chromatography was 632 $\pm$ 301 pg/ml. This was significantly higher (paired *t*-test, $t = 3.8$ $P < 0.01$) than the mean ($\pm$SD) of 423 $\pm$ 197 pg/ml obtained when chromatography was used, thus indicating that interfering substances were being removed by the inclusion of the chromatography step. Results obtained using the LH-20 system were validated by comparing them with those obtained following adsorption chromatography on alumina [12] in a further set of six samples. The mean ($\pm$SD) of 569 $\pm$ 169 pg/ml obtained after chromatography on LH-20 was not significantly different from that of 568 $\pm$ 167 pg/ml obtained following purification on alumina thus providing evidence of the adequacy of the

Table 1. Specificity of antiserum to androstenediol-7-cmo-BSA

Steroid	Cross reaction (%)
Androstenediol	100
Dehydroepiandrosterone (DHEA)	0.025
16α-Hydroxy DHEA	0.050
4-Androstene-3β,17β-diol	1.110
Testosterone	0.050
5α-Dihydrotestosterone	0.166
5α-Androstane-3β,17β-diol	6.050
Oestradiol	0.080
Pregnenolone	0.010

Per cent cross-reaction is expressed as the ratio of A/B × 100, where A = the amount of androstenediol and B = the amount of competitor required to reduce binding of [^{3}H]-androstenediol by 50%. Where, due to limited solubility of a steroid, this inhibition of binding was not possible, the ratio at the maximum inhibition of binding was used. DHEA sulphate, 5α-androstane-3α, 17β-diol, 5β-androstane-3α, 17β-diol, cholesterol, progesterone and cortisol all showed cross-reactions of less than 0.01%.

Table 2. Recovery of unlabelled androstenediol added to charcoal stripped plasma

Androstenediol added (pg/ml)	Mean androstenediol recovered (±SD) (pg/ml)	Number of determinations
100	90 (±16)	6
200	203 (±17)	6
400	384 (±21)	6
600	615 (±21)	4

LH-20 system. The interassay coefficients of variation [13] were 15%, 10% and 9% for the ranges of androstenediol 50–200 ($n = 24$), 201–400 ($n = 35$) and greater than 400 pg/ml ($n = 27$) respectively. Intra-assay variations for three quality control pools routinely included with 11 consecutive batches of plasma samples were 10%, 6% and 9% at mean levels of 227, 490 and 905 pg/ml respectively. Accuracy, measured by the recovery of unlabelled androstenediol added to charcoal stripped plasma was satisfactory (Table 2) and the sensitivity of the assay calculated from the means of duplicate determinations [13] was 40 pg/ml.

To establish a normal range, plasma androstenediol levels were measured in 113 normal women living on the island of Guernsey. The age range was 30–70 yr with a mean (±SD) of 50 ± 12 yr. Blood was taken by venepuncture between 2 p.m. and 8 p.m. and the heparinized plasma stored at −20° until analysed. The mean plasma androstenediol (±SD) for women between the ages of 30 and 50 (mean age 38 ± 8 yr) was 552 ± 223 pg/ml ($n = 49$) and for women between 51 and 70 yr (mean age 59 ± 5 yr) was 401 ± 217 pg/ml ($n = 64$).

The present approach of immunizing animals with androstenediol coupled through the C-7 position to the carrier protein has resulted in a very specific antiserum which cross-reacted to a negligible extent with steroids bearing the 5-ene, 3β-OH or 17β-OH functions, with the exception of 5α-androstane-3β, 17β-diol (6% cross-reaction). This steroid is very difficult to separate from androstenediol and neither chromatographic system described resolves them. Recently reported [14] mean levels of the androstanediol in the plasma of normal women (285 ± 67 pg/ml) indicate that interference from this steroid in the assay should be insignificant in the present study. However, in situations where the plasma level of 5α-androstane-3β, 17β-diol greatly exceeds that of androstenediol a chromatographic separation of the two steroids would be necessary for a specific assay of androstenediol.

Despite low cross-reactivity with other steroids a chromatographic step was essential for a valid assay. The major advantages of Sephadex LH-20 chromatography compared to most other chromatographic procedures are that the columns can be used several times and provided that the gel is thoroughly washed before use [14] its contribution to the blank value is negligible.

In the normal female, the major source of androstenediol is the adrenal cortex and the contribution of the ovary to plasma levels of this steroid is thought to be insignificant [2]. It is therefore unlikely that the stage of the cycle will affect the plasma androstenediol level and has been ignored in this study. The levels found in the premenopausal women are similar to those reported previously [1–3] and the tendency towards lower levels following the menopause is similar to that observed for other adrenal secretory products [15].

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