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5α-ANDROSTANE-3,17-DIONE IN PERIPHERAL PLASMA OF MEN AND WOMEN

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Summary—An antibody to androstanedione obtained in a rabbit by immunization with androstenedione-7α-carboxymethyl-thioether conjugated to bovine serum albumin was found to cross-react 100% with 5α-androstane-3,17-dione, a property that was used to develop a radioimmunoassay for this steroid. Plasma 5α-androstane-3,17-dione concentrations were determined in young men, and in women throughout an ovulatory cycle. In the men ($n = 6$), plasma 5α-androstane-3,17-dione concentrations were in the range of 84 to 273 pg/ml with a mean (±SD) value of 164 ± 57 pg/ml. The plasma levels in the women ($n = 5$) were in the ranges of 35 ± 14 to 145 ± 75 pg/ml during the follicular phase, and 109 ± 50 to 151 ± 44 pg/ml during the luteal phase. The tissue sites of origin of 5α-androstane-3,17-dione have not been defined, however some extraglandular tissues are known to contain enzymes that convert C_{19}-steroids to 5α-androstane-3,17-dione. It is possible that 5α-androstane-3,17-dione in circulation serves as a substrate for peripheral synthesis of 5α-dihydrotestosterone.

INTRODUCTION

The *in vitro* conversion of C_{19}-steroids, including dehydroepiandrosterone, androstenedione, testosterone, 5α-dihydrotestosterone, androsterone, and 5α-androstane-3α,17β-diol to 5α-androstane-3,17-dione (5α-A-dione) by human lung tissue, lung cells and epidermal keratinocytes has been demonstrated [1–8]. Moreover, 5α-A-dione is present in human prostatic tissue [9]. Based on these observations, we expected to find 5α-A-dione in human peripheral blood since the precursors for its synthesis are present in circulation [10]. 5α-A-dione was determined in human prostatic tissue by differential radioimmunoassay (RIA) by use of two antibodies—one that cross-reacted 100% with androstenedione and <0.1% with 5α-A-dione, and another that cross-reacted 100% both with androstenedione and 5α-A-dione [9]. Except for an abstract, presented without data, where it was stated that 5α-A-dione was determined by RIA in plasma of healthy subjects and patients with endocrine dysfunction [11], we are not aware of any other study on this subject. The availability of an anti-androstenedione antibody with high cross-reactivity for 5α-A-dione allowed us to develop a RIA for this steroid. With this technique we determined 5α-A-dione concentrations in peripheral plasma of men, and of women throughout the ovarian cycle.

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MATERIALS AND METHODS

Solvents, reagents, and glassware were as described previously [12]. 5α-A-dione and other non-radiolabeled steroids were purchased from Steraloids Inc (Wilton, NH). [1,2,6,7-^{3}H]Androstenedione (sp. act. 86 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [4-^{14}C]5α-Androstane-3,17-dione was synthesized [13].

The disposable glassware used in RIA was cleaned by heating in a self-cleaning oven. The celite was fired overnight in a muffle furnace at 800°C. Disposable serological pipets (5 ml) were used for preparation of chromatography mini-columns as described [14].

Blood collection

Venous blood (15 ml) was collected between 8 and 10 a.m. from men and women volunteers. Daily samples were obtained from five young women throughout a complete ovulatory cycle. Plasma was separated and kept frozen until assayed.

Hapten, antigen and antibodies

Androstenedione-7α-carboxymethyl-thioether was synthesized as described [15]. The conjugate of androstenedione-7α-carboxymethyl-thioether with bovine serum albumin, prepared by the carbodiimide method [15], was used for immunization of a 4-month-old New Zealand male rabbit, as described [12]. Rabbit serum prepared from blood obtained at the 3rd bleeding, 3 months after primary immunization (antiserum M43C-III), with high cross-reactivity for 5α-A-dione, was used for RIA.

Synthesis of [1,2,6,7-^{3}H]5α-A-dione

Purified nuclear membranes (10 mg protein) prepared from rat ventral prostate [16] were suspended in 0.1 M potassium phosphate buffer, pH 6.9, containing 0.25 M sucrose, NADPH (5 mM), and EGTA (1 mM) in a total volume of 2 ml; this mixture was transferred to a glass vial containing dried [1,2,6,7-^{3}H]androstenedione (9.1 μCi) and incubated at 24°C for 24 h, with shaking. The conversion of [1,2,6,7-^{3}H]androstenedione to [1,2,6,7-^{3}H]5α-A-dione was monitored by thin layer chromatography (TLC), as described below. The mixture was extracted with chloroform (3 × 10 ml), the pooled fractions were washed with water (1 ml), and evaporated to dryness with a stream of nitrogen. The residue was dissolved in isooctane (2,2,4-trimethylpentane, 0.5 ml); this solution was transferred to a mini-column prepared by packing tightly a mixture of celite (2-g)-ethylene glycol (1.5 ml) into a 5 ml disposable pipet and conditioned with isooctane (5.5 ml) [14]. Nitrogen pressure was used throughout the chromatographic procedure to force the eluant through the column. The tube was rinsed once with isooctane (0.5 ml) and the washing was transferred to the column. Elution was continued with isooctane (7 ml); fractions (0.5 ml) were collected and 10 μl aliquots were counted in a Packard Tri Carb Liquid Scintillation Spectrometer, Model 3300. The efficiency of tritium counting was 40%. The fractions containing [1,2,6,7-^{3}H]5α-A-dione were pooled and rechromatographed as above. The radiolabeled steroid, shown to be pure by TLC, was dissolved in ethanol for use in RIA.

Extraction of 5α-A-dione from human plasma for RIA

5 ml aliquots of male or female plasma were transferred to 25 × 150 mm teflon-capped glass tubes for solvent extraction. To each sample 25 μl of an ethanolic solution of [1,2,6,7-^{3}H]5α-A-dione (~4000 dpm, ~8 pg) was added as internal recovery standard. Identical aliquots were transferred to counting vials in triplicate for determination of total radioactivity added. The plasma samples were extracted with ethyl ether (20 ml) by shaking for 10 min in a Eberbach shaker (Ann Arbor, MI). After phase separation, the organic solvent extracts were transferred to clean tubes and evaporated to dryness under a stream of nitrogen; the residues, containing the extracted steroids, were dissolved in isooctane (0.5 ml) for use in chromatography, as follows.

Chromatographic purification of 5α-A-dione

Separation of 5α-A-dione from more polar steroids, including androstenedione, was achieved by column chromatography. The columns were prepared by packing firmly a mixture of celite (2.0 g) and ethylene glycol (1.5 ml) into 5 ml disposable serological pipetes and conditioning with isooctane (5.5 ml) [14]. The extracted steroids in isooctane were transferred to the columns, the tubes were rinsed once with isooctane (0.5 ml), and the washings transferred to the corresponding columns. Nitrogen pressure was used throughout the entire chromatographic procedure to force the solvent through the columns. The eluates were discarded and elution was continued with isooctane (4 ml). The first 1.5 ml eluted was discarded and the next 2 ml, containing 5α-A-dione, was collected for RIA.

RIA of 5α-A-dione

500 μl of the fractions containing 5α-A-dione were transferred to scintillation vials for counting to determine recoveries, and 500 μl aliquots were transferred to 12 × 75 mm glass tubes for RIA, which was conducted in a manner similar to that described previously for androstenedione [12], except for using 5α-A-dione and [1,2,6,7-^{3}H]5α-A-dione to obtain the standard curve. The final dilution of antiserum (M43 C-III) was 1:3500.

Specificity and sensitivity

The relative affinity of heterologous steroids for the antibody was determined by binding competition with [1,2,6,7-^{3}H]5α-A-dione, in a manner similar to that described by Thorneycroft *et al.* [17] for other steroids. The sensitivity

was determined by use of 3 standard deviations from the zero standard.

TLC of 5α-A-dione

After purification by column chromatography, the fractions containing 5α-A-dione in some selected samples were divided into aliquots to determine recoveries and for use in RIA; the remainders were purified further by TLC by use of Polygram Sil G/HY plastic plates (Brinckmann Instruments, Houston, TX) and benzene–ethyl acetate (9:1, 3 ascents) as the eluant. With this system, 5α-A-dione ($R_f = 0.67$) is separated completely from 5β-androstane-3,17-dione ($R_f = 0.55$).

The position of plasma 5α-A-dione on the thin layer plates was identified by running 5α-A-dione standard (10 μg) on the sides of the plates at positions widely separated from the experimental samples to avoid contamination. The lanes containing 5α-A-dione standard were cut out and sprayed with a mist of acetic acid, sulfuric acid, and *p*-anisaldehyde (100:2:1, per vol) and thereafter heated at 100°C for 20 min to visualize the steroid. The areas on the plates corresponding to 5α-A-dione were scraped, transferred to pasteur pipetes packed with glass wool, and eluted with ethyl acetate (5 ml). A 1 ml aliquot was used to determine recovery, and either 0.5 or 0.8 ml was used for RIA.

RIA of progesterone and LH

Plasma progesterone levels and immunoreactive LH in the 5 women were determined by RIA in a previous study [18].

Conversion factors to molar units

The conversion factor from pg/ml to pmol/l or ng/ml to nmol/l for 5α-A-dione is 3.571 and that for progesterone is 3.185.

RESULTS

A rabbit immunized with androstenedione-7α-carboxymethyl-thioether-bovine serum albumin—containing 7.2 molecules of hapten per molecule of protein [12]—produced an antibody (M43C-III) that had high cross-reactivity with 5α-A-dione (Table 1). Gradient elution column chromatography on celite–ethylene glycol [19] allowed the separation of 5α-A-dione from androstenedione (Fig. 1) and from most C_{19}-steroids present in plasma, including dehydroepiandrosterone, testosterone, 5α-dihydrotestosterone, androsterone, epiandrosterone, and etiocholanolone and, thus, we used this technique to separate 5α-A-dione from other steroids. 5β-Androstane-3,17-dione, progesterone, and 5α-pregnane-3,20-dione were not separated completely from 5α-A-dione (data not shown); the cross-reactivity of 5β-androstane-3,17-dione with the antibody was 4.1%, but the cross-reactivity with the C_{21}-steroids was <0.1% (Table 1).

The accuracy of the RIA was determined by two different procedures. In the first, known amounts of 5α-A-dione—in the range of 0.25 to 4 ng—were transferred to glass tubes and assayed: there was an excellent correlation between the amounts added and those determined by RIA (Fig. 2). In the second procedure, 5α-A-dione (0.3 ng/ml) was added to pooled blood bank plasma (5 ml) contained in 25 × 150 mm teflon-capped glass tubes. After extraction with ethyl ether and column chromatography, with and without further purification by TLC to ascertain the removal of 5β-androstene-3,17-dione, 5α-A-dione levels were determined by RIA. These levels were compared to those in corresponding plasma samples in the absence of added 5α-A-dione. The differences in 5α-A-dione levels between the samples where steroid was added and those without addition was 0.29 ng/ml after column chromatography alone and 0.27 ng/ml after both column chromatography and TLC (Table 2), values that correlate well with the amount added, i.e. 0.3 ng/ml. This finding is suggestive that 5β-androstane-3,17-dione, if present at all in plasma, does not interfere with the quantification of 5α-A-dione.

The sensitivity of the RIA was 7 pg/tube or 9 pg/ml (corrected for recoveries); the

Table 1. Cross-reactions of various steroids with antibody M43C-III raised against androstenedione-7α-carboxymethyl-thioether-bovine serum albumin conjugate

Steroid	Cross-reaction[a] (%)
Androstenedione	100
5α-Androstane-3,17-dione	100
5β-Androstane-3,17-dione	4.1
Androsterone	5.0
Epiandrosterone	9.5
5α-Dihydrotestosterone	0.6
Dehydroepiandrosterone	1.9
Estradiol-17β	0.1
Estrone	0.2
Progesterone	<0.1
5α-Pregnane-3,20-dione	<0.1
11β-Hydroxy-progesterone	<0.1
Pregnenolone	<0.1

[a] Calculated as:

$$\frac{\text{pg 5α-A-dione at 50\% binding}}{\text{pg heterologous steroid at 50\% binding}} \times 100.$$

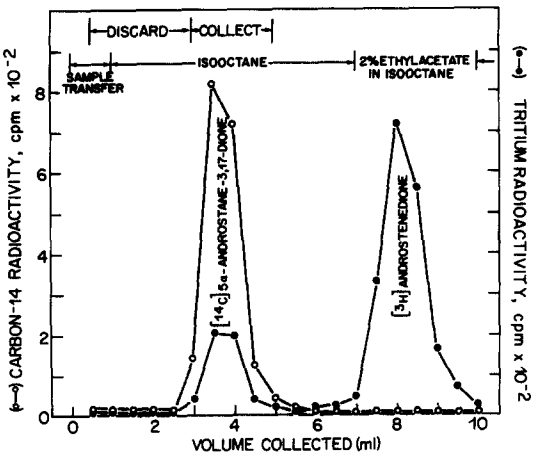

Fig. 1. Chromatographic separation. [4-^{14}C]5α-A-dione (~2000 cpm) was separated from [1,2,6,7-^{3}H]androstenedione (~2000 cpm) on a celite–ethylene glycol column by elution with isooctane, as described in the text. After discarding the transfer volume (2 × 0.5 ml) and the initial 1.5 ml of isooctane eluent, the next 2 ml contained ~98% of [4-^{14}C]5α-A-dione applied to the column. The bleeding of carbon-14 into the tritum channel was 30%. This chromatographic system was used to separate plasma 5α-A-dione from more polar steroids.

intra-assay variability, determined using plasma from a man with a 5α-A-dione level of 261 ± 17 pg/ml (mean ± SD, $n = 3$), was 6.5%, and the inter-assay variability ($n = 3$), obtained by use of the same plasma sample, was 10.4%. The water blank obtained by use of 5 ml of water, run in the RIA in parallel with the plasma samples, was undetectable.

The plasma levels of 5α-A-dione in 6 men were in the range of 84 to 273 pg/ml, with a mean (±SD) value of 164 ± 57 pg/ml (Table 3).

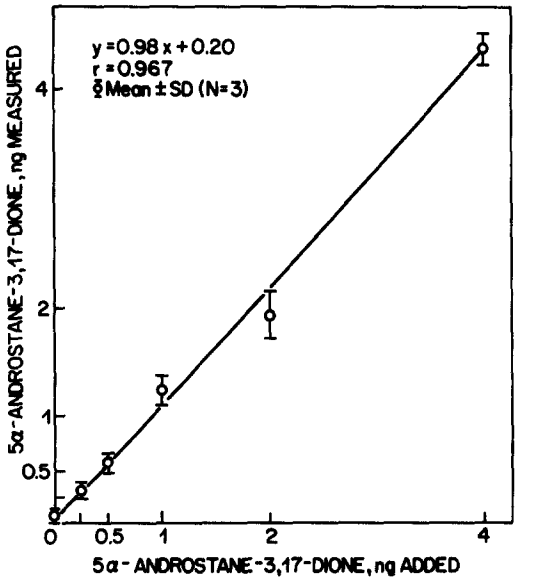

Fig. 2. Accuracy test. 5α-A-dione measured vs 5α-A-dione added.

Table 2. 5α-A-dione levels in human plasma, without and with 5α-A-dione addition[a]

n[b]	5α-A-dione added (0.3 ng/ml)	5α-A-dione measured (ng/ml) After column chromatography	After column and TLC
8	+	0.46 ± 0.04	0.48 ± 0.22
3	−	0.17 ± 0.03	0.21 ± 0.03

[a] Pooled blood bank plasma was divided into two aliquots: to the first, 5α-A-dione (0.3 ng/ml) was added while the second contained no additions. Replicate 5 ml aliquots were used for RIA. After the addition of [1,2,6,7-^{3}H]5α-A-dione (~4000 dpm) as internal recovery standard, samples were extracted and chromatographed on celite–ethylene glycol columns. Aliquots of the fractions containing 5α-A-dione were used to account for recovery and, also, for RIA. The remainders were evaporated to dryness and 5α-A-dione was purified further by TLC for use in RIA, as described in the text.

[b] n Represents the number of replicate samples.

In this study, RIA was conducted after purification of the steroid by either column chromatography alone or after both column chromatography and TLC (using 2 different aliquots). There was good agreement between the values obtained by use of these techniques (Table 3), a finding that supports the concept of absence of 5β-androstane-3,17-dione in human plasma.

The mean concentrations (±SD) of plasma 5α-A-dione in 5 young women determined throughout the follicular phase were in the range of 35 ± 14 to 145 ± 75 pg/ml and those determined throughout the luteal phase were in the range of 109 ± 50 to 151 ± 44 pg/ml (Table 4); the daily plasma 5α-A-dione levels are presented in Fig. 3. These women had a menstrual history suggestive of ovulation as demonstrated by their plasma progesterone and LH levels [18]. The time of ovulation in these women was estimated from the basal body temperature chart and the LH surge [20]. The day of the LH surge and the progesterone

Table 3. 5α-A-dione levels in men's plasma[a]

Sample No.	5α-A-dione (pg/ml) Column chromatography	Column chromatography and TLC[b]	Column chromatography and TLC[c]
1	133	108	131
2	182	193	226
3	117	171	129
4	126	84	100
5	273	269	242
6	152	144	171

[a] After addition of [1,2,6,7-^{3}H]5α-A-dione (~4000 dpm) to plasma samples (5 ml each) obtained from 6 men, steroids were extracted with ethyl ether (20 ml). After column chromatography to separate the fractions containing 5α-A-dione, aliquots were used to account for recovery and for RIA; the remainders were used for further purification of 5α-A-dione by TLC, as described in Materials and Methods.

[b] A 500 μl aliquot/5 ml ethyl acetate eluate (see Materials and Methods) was used for RIA.

[c] A 800 μl aliquot/5 ml ethyl acetate eluate was used for RIA.

Table 4. Plasma 5α-A-dione levels throughout the follicular and luteal phases in five ovulatory women

Subject	Ovarian phase	n^a	5α-A-dione (pg/ml)[b]
A.L.B.	Follicular	10	35 ± 14
	Luteal	18	148 ± 71
K.G.	Follicular	12	45 ± 26
	Luteal	15	109 ± 50
A.B.	Follicular	18	80 ± 58
	Luteal	15	143 ± 46
E.L.	Follicular	16	98 ± 35
	Luteal	13	116 ± 24
S.B.	Follicular	22	145 ± 75
	Luteal	10	151 ± 44

[a] n Represents the number of samples.
[b] Mean ± SD.

levels in corresponding plasma samples are presented in Fig. 3.

DISCUSSION

In vitro, 5α-A-dione is synthesized in various tissues from steroids precursors found in circulation [1–8]. To investigate whether 5α-A-dione was present in blood, we developed a specific RIA using an anti-androstenedione antibody that had high cross-reactivity with 5α-A-dione; a purification method that involved column chromatography on celite–ethylene glycol allowed the separation of non-polar 5α-A-dione from the great majority of steroids present in blood, except for 5β-androstane-3,17-dione, 5α-pregnane-3,20-dione, and progesterone. The cross-reactivity of the antibody with 5α-pregnane-3,20-dione and progesterone were minimal (<0.1%), but with 5β-androstane-3,17-dione it was 4.1%, however there were no differences in 5α-A-dione levels obtained after column chromatography alone or after further purification by TLC, which is indicative of the absence of 5β-androstane-3,17-dione in human plasma. Although various 5β-reduced pregnane and androstane metabolites are produced by the liver in relative large amounts, most of their precursors, viz. 5β-pregnane-3,20-dione and 5β-androstane-3,17-dione, do not appear to escape the liver cell or, if so, in very small amounts; these non-polar steroids undergo further metabolism *in situ* to produce the more polar compounds, which are excreted in urine either as the free steroids or in conjugated form. Thus, small amounts of 5β-pregnane-3,20-dione and 5β-androstane-3,17-dione have been reported to be present in human urine [21]. In human pregnancy plasma, however, 5β-pregnane-3,20-dione could not be detected by mass spectrometry [22] and it is possible that the same situation prevails with 5β-androstane-3,17-dione.

Plasma 5α-A-dione levels were determined in six men and five women. In the men, 5α-A-dione levels were in the range of 84 to 273 pg/ml. In the women, in general, the levels were slightly lower throughout the follicular phase than throughout the luteal phase. There was no correlation between plasma 5α-A-dione and progesterone levels determined in corresponding samples.

Plasma androstenedione levels in premenopausal women have been reported to reach a mean peak of 2.1 ng/ml at midcycle, and to fluctuate between 0.9 and 1.5 ng/ml at other phases of the ovarian cycle [10]. Plasma testosterone levels also are slightly elevated at midcycle, reaching a mean peak level of 0.5 ng/ml from basal levels of 0.2 to 0.3 ng/ml, but 5α-dihydrotestosterone levels fluctuate within

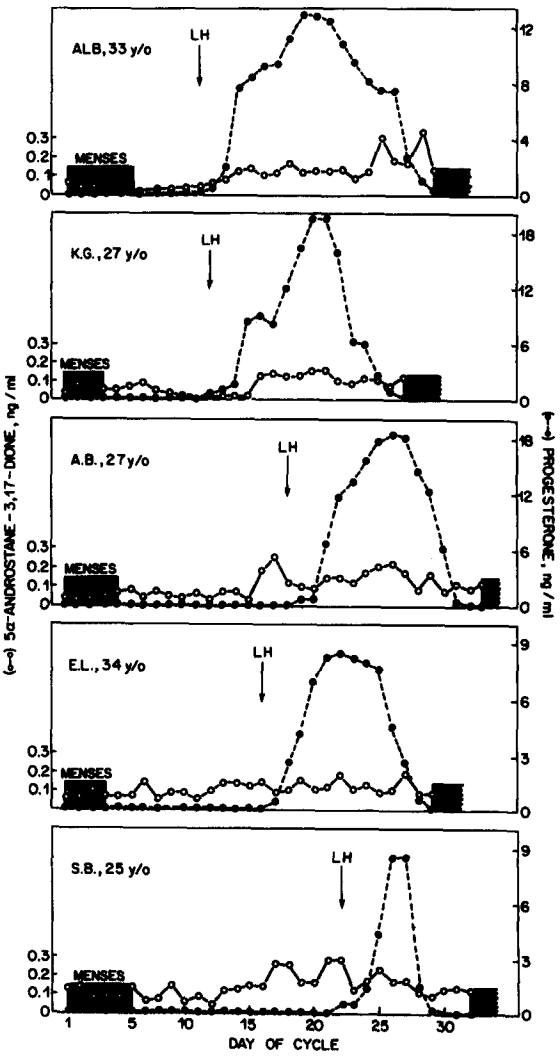

Fig. 3. Plasma 5α-A-dione levels in women. 5α-A-dione (○—○) and progesterone (●—●) levels determined daily in five women throughout the ovarian cycle.

narrow ranges throughout the cycle without a peak at midcycle, with mean values of 0.15 to 0.2 ng/ml [10]. In the women of our study, 5α-A-dione levels were similar to those reported for 5α-dihydrotestosterone [10].

We demonstrated previously that tissue 5α-reductase activity was not the limiting factor in the synthesis of 5α-pregnane-3,20-dione in women, but that substrate availability i.e. progesterone, regulate the plasma levels of this metabolite [18, 22]. The substrates for peripheral 5α-A-dione synthesis are present in plasma at relatively low levels, which may explain the low levels of this steroid in circulation.

5α-A-dione, administered subcutaneously, has androgenic properties in hypophysectomized-castrated rats that lead to significant weight increases in preputial gland and seminal vesicles as well as to increases in sebaceous gland mitosis [23], effects that occur via conversion to 5α-dihydrotestosterone. Thus, it is possible that in the human the physiological role of plasma 5α-A-dione is to serve as a precursor for peripheral synthesis of 5α-dihydrotestosterone.

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