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CONCENTRATIONS OF UNCONJUGATED 5α-ANDROSTAN-3β,17β-DIOL IN HUMAN PERIPHERAL PLASMA AS MEASURED BY RADIOIMMUNOASSAY

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SUMMARY

5α-androstane-3β,17β-diol was measured in human peripheral plasma using a specific antibody generated against a carboxymethyloxime BSA conjugate linked at position 7. Concentrations were significantly higher in normal men than women. Preliminary results suggest that plasma 5α-androstane-3β,17β-diol concentrations might be a useful clinical parameter in cases of hirsutism and male infertility.

INTRODUCTION

Although 5α-androstane-3β,17β-diol (3β diol) is a weak androgen as assessed by classical bioassay methods [1], recent work suggests that it may have considerable physiological importance. A role has been proposed for 3β diol in the initiation of puberty in the female rat [2]. Additionally, 3β diol is secreted by the rabbit testis [3], its secretion being significantly reduced in artificial cryptorchidism whereas that of testosterone, dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α diol) was unaffected. Controversy exists in the literature concerning the role of 3β diol in prostate physiology [4, 5]. We considered it would be worthwhile developing a rapid, specific radioimmunoassay for 3β diol to assess its importance in human reproductive physiology. A suitable conjugate was therefore synthesised, antisera raised and characterised and an assay system developed and validated for human peripheral plasma.

MATERIALS AND METHODS

[1,2-^{3}H]-5α-Androstane-3β,17β-diol 43 Ci/mmol was obtained from the Radiochemical Centre, Amersham. 3β,17β-Diacetoxy-5-androstene was purchased from Koch Light Laboratories, Colnebrook, England, 5β-androstane-3β,17β-diol from Ikapharm, Israel and all other steroids were from Steraloids Inc., Pawling, USA except 5α-androstane-3β,17α-diol which was a gift from the MRC reference steroid collection. Tris gel consisting of 0.05 M Tris pH 8.5 containing 0.1% (w/v) gelatine was used for all dilutions. Charcoal (0.25 g Norit A, Sigma) was suspended in 100 ml Tris gel containing 2.5 mg Dextran T40 (Pharmacia). General reagents (Analar grade) were obtained from B.D.H., Poole, England (for preparations of antigen) and from Merck, Darmstadt, Germany (for radioimmunoassay work). All solvents were distilled prior to use, diethyl ether over ferrous sulphate. Freud's complete adjuvant was from Difco Laboratories, U.S.A.

Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. I.R. spectra were determined from mulls in Nujol on a Unicam SP 200 spectrophotometer. Ultraviolet spectra were determined on a Unicam SP 800 spectrophotometer. Nuclear magnetic resonance proton spectra were recorded on a Varian HA 100 spectrometer from solutions in deuterated dimethyl-sulphoxide. Mass spectra for the synthesis work were recorded on an A.E.I. MS 12 spectrometer and only the molecular ion (M^+) is quoted.

Scintillator was from Roth, Karlsruhe, Germany and radioactivity was counted in a Beckman Model LS-250 Liquid Scintillation Counter. Silylation was performed with *N*-methyl-trimethyl-silyl-trifluoroacetamide–trimethylchlorosilan 99:1 from Macherey & Nagel, Düren, Germany.

Synthesis of antigen

3β,17β-Diacetoxy-5-androsten-7-one. To a well-stirred solution of 3β,17β-diacetoxy-5-androstene (3.6 g) in dichloromethane (distilled from calcium hydride, 250 ml) under an atmosphere of dry nitrogen was added chromium trioxide–pyridine complex (42 g) [6] in five portions over two days. The reaction mixture was then poured onto ether (100 ml) and the tarry residue washed with ether (100 ml × 8). The combined ether extracts were washed with saturated

aqueous sodium bicarbonate (100 ml × 12), dried (Na_2SO_4) and concentrated by rotary evaporation. Recrystallisation of the residue from ethyl acetate–hexane gave 3β,17β-diacetoxy-5-androsten-7-one as white prisms (3.1 g) m.p. 217–219°C, lit. m.p. [7] 216–219°C.

3β,17β-Diacetoxy-5α-androstane-7-one. A solution of the above enone (2.2 g) in ethyl acetate (60 ml) containing pyridine (0.2 ml) and 10% palladium on charcoal (0.15 g) was stirred under an atmosphere of hydrogen for 4 h. After flushing with nitrogen, the solution was filtered through celite, concentrated and the residue crystallised from ethyl acetate–hexane to give 3β,17β-diacetoxy-5α-androstane-7-one (2.1 g) as colourless plates, m.p. 190–192°C, lit. m.p. [7] 192–193°C. Chromatographic analysis of the product showed that a minor by-product formed in the reaction, possibly the 5β-isomer, had been removed by crystallisation.

3β,17β-Diacetoxy-5α-androstane-7-one 7-(O-carboxymethyl)oxime. A solution of the above ketone (1.2 g) and O-(carboxymethyl) hydroxylamine hemihydrochloride (0.48 g) in anhydrous pyridine was stirred at 25°C for 24 h. Removal of the pyridine in a stream of dry nitrogen left an oil which was partitioned between ethyl acetate (100 ml) and M hydrochloric acid (50 ml). The organic phase was washed with saturated brine (50 ml × 2), dried (Na_2SO_4) and concentrated. Recrystallisation of the residue from ethyl acetate–hexane gave 3β,17β-diacetoxy-5α-androstane-7-one 7-(O-carboxymethyl)oxime as white plates (1.1 g) m.p. 186–188°C. I.R. μ_{max}; 1725 (acetates) and 1700 cm^{-1} (carboxyl) n.m.r. δ(d_6-DMSO); 0.62 (3H, S, 18-CH_3), 0.90 (3H, S, 19-CH_3), 2.52 (6H, S, $OCOCH_3$) and 4.43 (2H, S, OCH_2CO). Mass spectrum: M^+ 463 ($C_{25}H_{37}NO_7$ requires 463).

3β,17β-Dihydroxy-5α-androstane-7-one 7-(O-carboxymethyl)oxime. A solution of the above diacetate (1.0 g) in methanol (160 ml) and 8 M sodium hydroxide (40 ml) was stirred under an atmosphere of nitrogen at 30°C for 24 h. The solution was adjusted to pH 3 with formic acid and extracted with ethyl acetate (100 ml × 3). The combined extracts were washed with saturated brine (50 ml × 3), dried (Na_2SO_4) and concentrated. Crystallisation of the residue from aqueous ethanol gave 3β,17β-Dihydroxy-5α-androstane-7-one 7-(O-carboxymethyl)oxime as white prisms (0.59 g) m.p. 158–160°C. I.R. ν_{max}; 3400, 3300 (hydroxyls); 1700 (carboxyl) and 1640 cm^{-1} (imine) n.m.r. δ(d_6-DMSO); 0.70 (3H, S, 18-CH_3), 1.10 (3H, S, 19-CH_3) and 4.43 (2H, S, OCH_2CO). Mass spectrum: M^+ 379 ($C_{21}H_{33}NO_5$ requires 379).

The derivatised steroid was coupled to BSA as previously described [8]. The molar steroid:protein ratio of the conjugate was determined, using nitropropane [9], to be 28.

Immunisation schedule

Two New Zealand White rabbits were used for the immunisation. An initial dosage of 2 mg antigen per rabbit was given, distributed among several sites along the back. Two weeks later this was repeated and thereafter booster injections were given using 1 mg of the antigen at the 9th, 15th and 21st weeks. All injections were made up in 1 ml of saline emulsified with 1 ml of Freund's complete adjuvant. Animals were bled periodically at around 14 days after the booster injections. At one stage (after the 2nd booster injection) animals were bled at three day intervals. Titre was assessed by incubating 200 μl diluted antibody with 30,000 d.p.m. (0.32 pmol) β diol in 100λ Tris gel at 4°C overnight and proceeding as described for the radioimmunoassay.

Radioimmunoassay

Approximately 1000 c.p.m. [1,2H^3]-3β diol in 50 μl methanol was added to serum sample aliquots and the mixture allowed to equilibrate at room temperature for a minimum of 30 min. Two different aliquots (usually 1 ml and 2 ml) were used for each sample. Serum was extracted twice with four vol. diethyl ether and the combined extracts evaporated to dryness under nitrogen. The dried extract was transferred to a 7 cm long, 0.5 cm diameter Sephadex LH20 column head in 2 × 100 μl benzene–methanol (95:5, v/v), the latter also being used to pack and elute the column. The first 4.5 ml eluate were discarded, the next 2.5 ml collected and evaporated to dryness under nitrogen. The small columns were used repeatedly, being washed between assays by 0.4 ml benzene–methanol (50:50, v/v) followed by 10 ml benzene–methanol (95:5, v/v). The dried extract was taken up in 200 μl Tris gel and, after being allowed to stand for 10 min, 100 μl and 50 μl aliquots were removed for assay and recovery estimation respectively. 100 μl diluted antibody (1:3000 in Tris gel) was pipetted into the assay tubes. A standard curve was simultaneously prepared from solutions containing 50–1000 pg per 100 μl Tris gel. After 1 h incubation at room temperature (20°C), 10,000 c.p.m. tritiated 3β diol in 100 μl Tris gel was added and the tubes incubated overnight at 4°C. Bound steroid was separated from free using the dextran coated charcoal method. The supernatant was decanted, 10 ml scintillator added and the radioactivity counted. Approximately 35% of labelled steroid was bound in the zero tubes. Blanks were prepared in a manner identical to the samples except that water was extracted instead of serum. Results were calculated after logit-log transformation of the data. The effective range of the standard curve was from 20 to 500 pg, 50% displacement being obtained with about 150 pg.

In some samples, testosterone was measured as previously described [10] and FSH using the Serono kit with values given in mIU/ml 2nd IRP, normal range for men being 5–20 mIU/ml.

Estimation of 3β diol in a serum pool by GLC–mass fragmentography

After addition of 3000 c.p.m. [H^3]-3β diol, 20 ml

plasma was extracted four times with three vol. diethyl ether and evaporated to dryness under nitrogen. The extract was then chromatographed using the Sephadex LH20 column as described above. The use of such columns for purification prior to GC-MS has been recommended by Ganjam *et al.* [11]. An aliquot of the 3β diol fraction was removed and the radioactivity counted to estimate recovery.

GLC–mass fragmentography was performed on a Varian MAT 711 mass spectrometer equipped with a Varian 2700 gas chromatograph and a two stage Watson-Beaman separator. A 1.6 m × 1/8″ column with 3% SE 30 on Chromosorb W/AMDMCS 80–100 mesh at 220°C and with helium pressure of 28 psi was used. MS data: SEV 2.5 kV, 0.4 mA emission, 70 eV, inlet 250°C.

The gas chromatograph was recorded by the total ion current. The retention times were: 5α-androstane-3β,17β-diol 7.5 min; 5α-androstane-3α,17β-diol 6.1 min; 5β-androstane-3α,17β-diol 6.2 min; 5β-androstane-3β,17β-diol 7.0 min; 5-androstene-3β,17β-diol 6.9 min. The mass spectrum of 3β diol showed intensive peaks at *m/e* 436, 421, 346 and 241, the first two ions being used in single ion mode determination by external standardisation. Standards consisting of 0.15, 0.20, 0.28, 0.40 and 0.80 ng/μl gave a linear peak height response; pool sample content of 3β diol was determined by comparison of peak heights. The final concentration of 3β diol in the plasma was calculated after correction for losses.

Sampling procedures

Blood samples were taken from patients and volunteers during morning hours by venipuncture and allowed to clot before centrifugation at 4°C. Serum was stored at −20°C until required for assay. Normal women were adult, premenopausal, cycling and were not receiving oral contraceptive medication. Samples were taken at random with regard to stage of the menstrual cycle. Two adult male patients were castrated, one because of testicular tubercular lesions and the other because of testicular abcess. One of these patients was subsequently treated with testosterone esters injected intramuscularly. With the exception of the latter, patients were not receiving medication at the time of sampling. A sample of follicular fluid was obtained from a large follicle from a 52-year-old premenopausal woman at operation for hysterectomy.

RESULTS

Both immunised rabbits responded and titres were 1:1000 (R10) and 1:5500 (R11) 13 weeks after primary immunisation. Titres increased to about 1:8000 after 26 weeks. The specificity of the two antibodies is given in Table 1. It can be seen that both antibodies were highly specific with respect to all steroids tested except 5-androstene-3β,17β-diol. Because 3β diol and 5-androstene-3β,17β-diol are not easily separated chromatographically, antibody R10 was selected for use in the radioimmunoassay. Assay specificity was evaluated by adding known amounts of potential interfering substances to 1.0 ml water and proceeding as described above. 10 ng testosterone, DHT, dehydroepiandrosterone (DHA) and 1 ng 5-ene-androstenediol gave values indistinguishable from blanks. By contrast, 3β diol was quantitatively recovered (see Table 2a). In addition, the 3β diol content of a plasma pool was estimated by radioimmunoassay and by GLC mass fragmentography giving values of 444 ± 26 pg/ml (S.D., $n = 5$) and 463 pg/ml (mean of 3 determinations) respectively. Chromatography was

Table 1. Cross-reactivity data on antisera to 5α-androstane-3β,17β-diol-7-CMO-BSA

	R10	R11
5α-androstane-3β,17β-diol	100	100
5β-androstane-3β,17β-diol	<0.1	0.5
5-androstene-3β,17β-diol	2.9	10.1
4-androstene-3β,17β-diol	<0.1	<0.1
5α-androstane-3α,17β-diol	0.2	<0.1
5α-androstane-3β,17α-diol	—	<0.1
5β-androstane-3α,17β-diol	0.2	<0.1
5α-androstane-3β-ol	—	<0.1
DHA	<0.1	<0.01
Testosterone	0.01	<0.1
5α-DHT	0.5	0.45
Epiandrosterone	0.32	<0.1
4-androstenedione	<0.1	<0.1
5α-androstanedione	<0.1	<0.1
Pregnenolone	<0.01	<0.01
Progesterone	<0.01	<0.01

Cross-reactions were determined as the ratio, expressed as a percentage, of concentrations of the steroid in question to 3β diol required to reduce binding by 50%. When this was not possible due to limited solubility of certain steroids, the steroid: 3β diol ratio at maximum obtainable binding inhibition was used.

Table 2. a. Accuracy

3β Diol added (pg)	3β Diol recovered (pg)	
	From water	From plasma
100	100	86
200	180	208
500	421	
1000	954	830

b. Plasma parallelism and effect of chromatography omission

Plasma vol. (ml)	Value with chromatography (pg/ml)	Value omitting chromatography (pg/ml)
0.2	411	
0.4	472	842
1.0	459	613
2.0		534

Values given are the means of duplicates. The plasma used for both the recovery and parallelism experiments was a pool consisting of a mixture of samples from female patients.

Table 3. Peripheral serum concentrations of 3β diol

	3β Diol concentrations ± S.D. (pg/ml)
Normal men	607 ± 219 (17)
Normal women	285 ± 67 (11)
Hirsute women	585 ± 53 (6)
3rd trimester pregnant women	313 ± 127 (9)
Individual patients:	
Human follicular fluid	725
Castrated adult man, untreated	200
Above patient after injection of testosterone esters (testosterone 10 ng/ml)	220
Castrated adult man, untreated	236
Severe oligozoospermia* (testosterone 6.0 ng/ml, FSH 5.1 mIU)	551
Sertoli cell only syndrome (testosterone 5.0 ng/ml, FSH 25 miU)	295

The number of cases is shown in parentheses.

* This patient was subsequently successfully treated with gonadotrophins, the sperm count rising from 0.4×10^6 to 24×10^6 per ml.

however essential for assay validity as shown in Table 2b. The within-assay coefficient of variation at 90 pg/tube was 14% ($n = 7$) and at 400 pg/tube 8% ($n = 5$). The blank value was 18 ± 9 pg giving a sensitivity of about 40 pg. Values are shown in Table 3.

The 3β diol concentration sex difference was significant and castrated men had values in the low normal female range. The level in the follicular fluid sample was approximately three times that of plasma. The 3β diol level of the male infertility patient with a normal FSH value was within the normal range whereas that of the Sertoli cell only syndrome patient with an elevated FSH was subnormal. Levels were elevated in hirsute women.

DISCUSSION

Although it has been possible for some time to measure 3β diol in plasma, previous procedures were too slow and tedious to permit estimation of this steriod in a large number of plasma samples. Schanbacher and Ewing[12] determined 3β diol in rabbit plasma using an antibody against a testosterone–BSA conjugate which cross-reacted with 3β diol, 3α diol and DHT. The disadvantage here is the time-consuming chromatography needed to separate the diol epimers, a procedure obviated here by the specificity of the antibody. Chromatography was, however, found to be essential for assay validity, probably due to non-specific interference from plasma lipids. Kohen and Lindner[13] have prepared an antibody to 3β diol linked to BSA in position 15. This antibody, although having a negligible cross-reaction with 5-androstene-3β,17β-diol, cross-reacted 13% with DHT and since the latter and 3β diol are present in men and women in approximately equal concentrations, chromatography would presumably be necessary here too, even if this antibody was insensitive to non-specific interference. Assay specificity appeared satisfactory as would be expected from the cross-reaction data. Blood levels of 5-ene-androstene-3β,17β-diol have been reported to be similar to the levels of 3β diol given here [14] and interference would thus not be expected to be serious although these steroids are not resolved by the Sephadex LH20. The method would not be suitable for samples having a very high ratio of 5-androstene-3β,17β-diol to 3β diol.

Normal men had significantly greater concentrations of 3β diol than normal women, as seems to be the case for all androgens hitherto studied. The value for normal women is in accord with the preliminary results of Murphy [15], who measured plasma androgens by competitive protein binding after separation using 31 cm columns of Sephadex LH20. The two castrated patients had levels approximately 40% those of intact men, suggesting that the testis contributes approximately 60% of the 3β diol plasma pool (including both direct secretion and peripheral conversion of other testicular hormones). No increase was noted when one of these patients received intramuscular testosterone esters. Peripheral conversion of testosterone is probably not a major source of plasma 3β diol, very low conversion rates having been reported in men and women [16]. This conclusion is also in agreement with previous reports demonstrating on the one hand a four to five fold greater conversion of both testosterone and DHT to 3α rather than 3β diol [16], and lower plasma values of the former than the latter epimer [17] on the other. Both epimers have the same SBG binding affinity [18], suggesting that differences in clearance rates would not account for these observations. The conversion of DHA and 5-androstene-3β,17β-diol to 3β diol by pathways not passing through testosterone has been demonstrated in man [19].

Follicular fluid contained only three times the 3β diol of plasma and although this result does suggest

that the ovary is indeed a source of 3β diol, its relative contribution in normal women is probably very small. The low concentration of 3β diol in follicular fluid contrasts with concentrations of testosterone, oestradiol and progesterone which have been reported to be around 50 ng/ml, 100 ng/ml and 1 μg/ml respectively [20]. Third trimester pregnant women had levels only slightly and not significantly greater than non-pregnant values. This would indicate that the placenta, at least in the third trimester, is not an important source of 3β diol, which agrees with a previous report in which very little 3β diol was formed from DHA by human placental tissue *in vitro* [21].

All six hirsute women assayed had elevated levels of 3β diol similar to those found in males. Three of these patients had serum testosterone levels within the normal female range suggesting that other androgens such as 3β diol might be important in hisutism, as has been suggested by Rosenfield[22]. Increased conversion of both testosterone and DHT to 3α and 3β diol in hirsute women have been reported [16] and 3β diol is a powerful stimulator of sebum production in rats [23]. Plasma 3β diol might therefore be a useful clinical parameter in cases of hirsutism and acne, regardless of whether 3β diol is active in this respect *per se*, or whether it is an inactive end product reflecting increased utilisation of other androgens by the skin [24].

Insufficient numbers of male infertility samples have been assayed to allow definite conclusions to be drawn about any possible role of 3β diol in human spermatogenesis. The reduced level of plasma 3β diol but not testosterone in the Sertoli cell only syndrome patient (the only male patient, excepting the castrates, with <400 pg/ml) is interesting in the light of previous work in which formation of 3α and 3β diol by human seminiferous tubules was far greater than by interstitial cells [25].

The physiological importance of unconjugated 3β diol in man, in particular whether it is an active androgen *per se* in physiological concentrations or is active only after conversion to DHT [4, 5] is unknown. The availability of a rapid, specific radioimmunoassay should throw light on the role of 3β diol in normal and pathological physiology and could perhaps be of clinical value.

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