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DETERMINATION OF 5α-ANDROSTANE-3α,17β-DIOL AND 5α-ANDROSTANE-3β,17β-DIOL IN HUMAN PLASMA BY RADIOIMMUNOASSAY

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Summary—5α-Androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3β-diol) were measured in human peripheral plasma by radioimmunoassay using celite microcolumn purification. The antisera used for the assay were obtained by immunization of rabbits with 3α,17β-dihydroxy-5α-androstane-6-(*O*-carboxymethyl) oxime: BSA for 3α-diol and 3β,17β-dihydroxy-5α-androstane-15α-carboxymethyl: BSA for 3β-diol. The concentrations (pg/ml ± SD) of the two diols in normal male and female plasma are respectivly:

216 ± 51 and 49 ± 32 for 3α-diol,
239 ± 76 and 82 ± 45 for 3β-diol.

Comparison of these results with published ones shows that 3β diol concentrations were significantly lower. The high specificity of the assay is due to chromatography on celite microcolumns, allowing elimination of 5-androstene-3β,17β-diol from the plasma sample.

INTRODUCTION

Testosterone is metabolised in target tissues into 5α-dihydrotestosterone through 5α-reduction, followed by reduction at C-3 leading to two epimers, 5α-androstane-3α,17β-diol and 3β,17β-diol. 3α-Diol, which is a potent androgen, is an end product of testosterone metabolism, while its epimer 3β-diol, a weak androgen, is metabolised *in vivo* to 3α-hydroxylated compounds (androsterone, 3α-diol) and to tri-hydroxylated metabolites (2α- and 7α-hydroxylation) [1]. The determination of these two diols in plasma is a necessary complement of urinary assays [2]. Many authors [3–11] determined 3α- and 3β-diol levels in human plasma by radioimmunoassay. But as the purification of the plasma samples seemed unsatisfactory, chromatography on celite microcolumns was used in the present investigation. This chromatographic step allows a separation of 3α-diol, 3β-diol and 5-androstene-3β,17β-diol, a compound present in plasma at a level of 1 ng/ml [12–15]. Specificity of the method was checked by high performance liquid chromatography purification of samples before quantification by radiodimmunoassay.

EXPERIMENTAL

Materials

Chemicals. Solvents and reagents of analytical grade are not purified prior to use. Each batch of solvents and chromatographic material are checked to estimate the blank value. [1α,2α-^{3}H]5α-Androstane-3α,17β-diol (sp.act. 40 Ci/mmol), [1α,2α-^{3}H]5α-androstane-3β,17β-diol (sp.act. 45 Ci/mmol) were purchased from the Radiochemical Centre, Amersham (England). The radiochemical purity of these compounds was checked by thin layer chromatography. 3α-Hydroxy-androst-5-en-17-one (dehydroandrosterone) was purchased from Merck, Darmstadt (Germany). Celite was purchased from Touzart et Matignon and heated at 500°C during 16 h before use. The sodium phosphate buffer, pH 7, contained 5.38 g NaH_2PO_4, H_2O (50 mM), 21.84 g Na_2HPO_4, 12 H_2O (60 mM), 9 g NaCl, 1 g NaN_3, 2 g gelatin per liter of distilled water. Scintillant fluid was made for 12.5 g 2,5-diphenyloxazol (P.P.O.) and 1.25 g 2,2′-*p*-phenylenbis (4-methyl-6-phenyloxazol) (POPOP), per 2.5 l of toluen (Merck). Radioactivity was counted in an Intertechnique Model SL 4000 spectrometer.

Antigens. 3β,17β-Dihydroxy-5α-androstane-15α-carboxymethyl:BSA was a gift from Dr Roméo Emiliozzi (University of Nice). 3α,17β-Dihydroxy-5α-androstane-6-(*O*-carboxymethyl oxime):BSA was prepared from dehydroandrosterone. Reduction of ketone at C 17 leads to 5-androstene-3α,17β-diol. Nitration with sodium nitrite, acetic and nitric acid followed by reduction with zinc, acetic acid–water medium [16] of 5-androstene-3α,17β-diacetate leads to a mixture of 3α,17β-diacetoxy-5α- and -5β-androstan-6-one (ratio 5α/5β = 7/3). After saponification and alumina column chromatography,

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the steroid was derivatized (6-CMO) and coupled to BSA [17].

Antisera. 3α-Diol antiserum produced in rabbits immunized with 3α,17β-dihydroxy-5α-androstane-6-(*O*-carboxymethyl oxime):BSA was diluted 1/1000 for the determination of 3α-diol. 3β-Diol antiserum produced in rabbits immunized with 3β,17β-dihydroxy-5α-androstane-15α-carboxymethyl : BSA was diluted 1/2000 for the determination of 3β-diol.

Methods

Extraction. Twenty μl of a solution of [1α, 2α-^{3}H] 3α- or 3β-diol (700 dpm viz 12 Bq) in phosphate buffer were added to plasma (3 ml). Samples were allowed 1 h at room temperature. Plasma was extracted with 10 ml of diethylether, with an automatic stirrer (Sybron, Thermolyne Maxi Mix) for 30s. The agitation was repeated twice. After freezing, the organic layer was saved and evaporated to dryness under nitrogen stream.

Chromatography. Celite, after activation at 500°C for 16 h, was gradually cooled and then impregnated with ethylene glycol (2:1, w/v) according to Abraham's method [18]. Glass microcolumns (0.6 cm internal dia) were packed with 0.75 g of impregnated celite. Six ml of isooctane were passed through the column and discarded. Plasma extract was dissolved three times with 0.2 ml of isooctane and transferred to the celite microcolumns. 4.5 ml of isooctane were passed through the column and discarded. The DHT and T fraction was then eluted with 9 ml isooctane–benzene (90:10, v/v) and discarded. The 3α-diol fraction was eluted with 7 ml isooctane–benzene (90:10, v/v), collected and evaporated to dryness. The 3β-diol fraction was eluted with 9 ml of the same solvent mixture, collected and dried under nitrogen stream. Elution diagram is shown in Fig. 1. Elutates were dissolved in 1 ml ethanol, 0.1 ml was used for recovery determinations.

Radioimmunoassay. Standard curves were prepared with 5, 10, 25, 50, 100, 150, 200 pg of unlabelled 3α- or 3β-diol. 3000 dpm of the corresponding tritiated steroid and 200 μl antiserum were added into the standard and the assay tubes ($\frac{1}{10}$ and $\frac{1}{5}$ of the sample). The tubes were then incubated at 37°C for 30 mn and thereafter at 18°C for 30 mn. The free steroid was separated from the bound by extraction with 3 ml of scintillant fluid, according to Castanier *et al.* [19]. The organic layer was transferred in polypropylene tube and radioactivity counted. Sufficient counts were accumulated so that the per cent deviation of statistical error did not exceed ± 2% at the 95% confidence level. Standard curves are shown in Fig. 2.

RESULTS

Recovery

Following extraction, the mean per cent recovery for 3α- and 3β-diol were respectivly 94 ± 6 and 94 ± 5 ($n = 20$). After chromatography on celite microcolumns, the mean per cent recovery for the two diols were respectively 58 ± 11 and 65 ± 10 ($n = 20$).

Blank values

Blank values evaluated on phosphate buffer (3 ml) extracted and chromatographied as a plasma sample showed 9 ± 2 pg/tube ($n = 20$) for 3α-diol and 14 ± 2 pg/tube ($n = 20$) for 3β-diol assay.

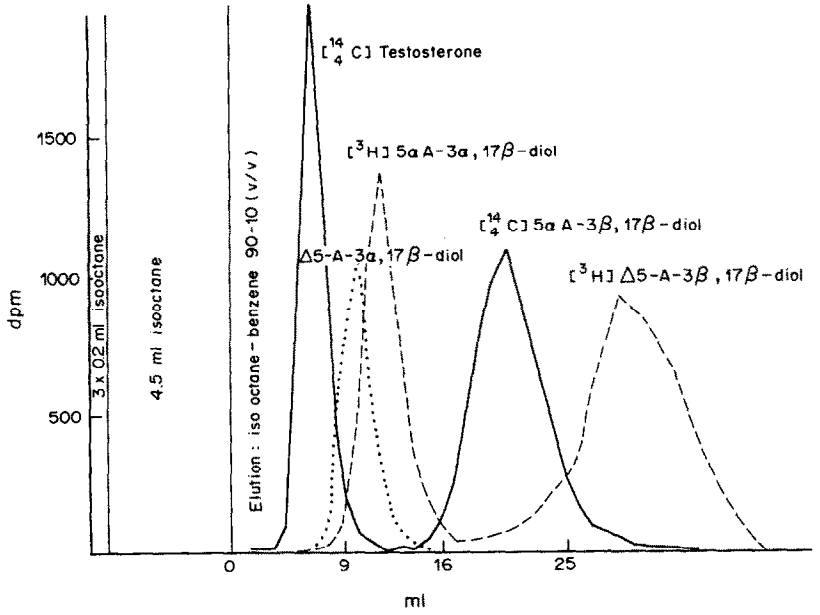

Fig. 1. Elution of testosterone, 5α-androstane-3ξ,17β-diol and 5-androstene-3ξ,17β-diol on celite microcolumn (the detection for 5-androstene-3α,17β-diol was performed by Gas Chromatography as trimethylsilyl ether derivatives).

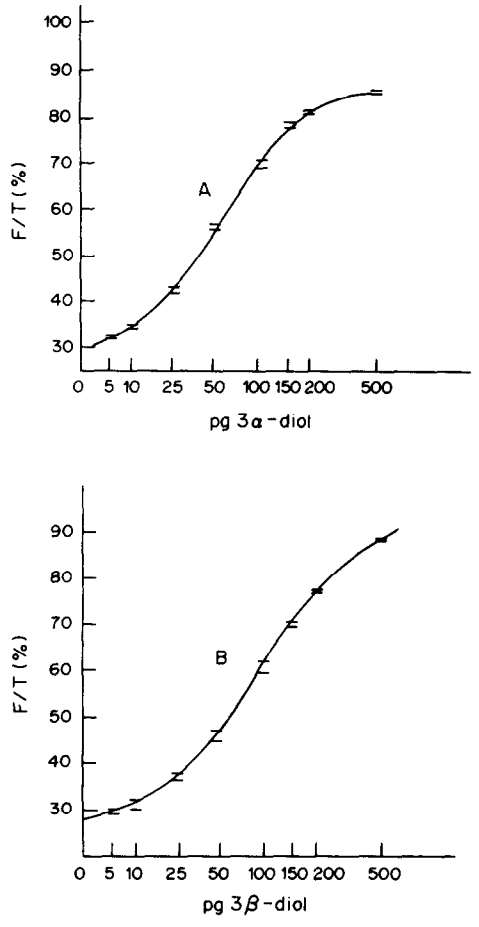

Fig. 2. Standard curves for radioimmunoassay of 5α-androstane-3ξ,17β-diol. *A*: 5α-Androstane-3α,17β-diol assay. 3α-Diol antiserum produced in rabbits immunized with 3α,17β-dihydroxy-5α-androstane-6-(*O*-carboxymethyl) oxime:BSA was diluted 1/1000 for the assay.

B: 5α-Androstane-3β,17β-diol assay. 3β-Diol antiserum produced in rabbits immunized with 3β, 17β-dihydroxy-5α-androstane-15α-carboxy methyl:BSA was diluted 1/2000 for the assay.

Accuracy

150, 300, 600, 1500 pg of 3α-diol and 150, 300, 1000, 1500 pg of 3β-diol were added to 3 ml of plasma stripped of steroids by charcoal treatment, and measured n times ($4 \leqslant n \leqslant 8$). The correlation curves are shown in Fig. 3.

Precision

Intra-assay precision was determined by measuring 3α- and 3β-diol concentrations of the same plasma pool in ten different assays on the same day and with the same calibration curve. The coefficients of variation were 11.5% for 3α-diol and 19% for 3β-diol. The two diols levels were measured on the same plasma pool, at two different days, with two different calibration curves. 3α-Diol concentration was evaluated to 119 ± 22 pg/ml ($n = 10$) for the first assay and to 122 ± 14 pg/ml ($n = 10$) for the second assay. For 3β-diol, the results were respectively 140 ± 27 pg/ml ($n = 10$) and 151 ± 35 pg/ml ($n = 10$).

Table 1. Specificity of the antisera:cross reactions (%) by Abraham's method

Steroid	3α-Diol antiserum (1:1000)	3β-Diol antiserum (1:2000)
5α-A-3α,17β-diol	100	1
5α-A-3β,17β-diol	0	100
5-Androstene-3α,17β-diol	21.6	—
5-Androstene-3β,17β-diol	—	37
5β-A-3α,17β-diol	4.2	0.6
Testosterone	0.07	0.5
5α-DHT	0.1	2
Androsterone	5.7	—
Epiandrosterone	—	2
Progesterone	0.12	0
Estradiol	0	0.1

Detection limit

According to Currie [20], three levels must be distinguished.

L_C: critic decision limit = $1.645\sigma_{bl}$

L_D: detection limit = $3.29\sigma_{bl}$

L_Q: determination limit: $10\sigma_{bl}$

where σ_{bl} represented the standard deviation of the blank value. For 3α- and 3β-diol, L_C, L_D and L_Q were respectively evaluated at 3.29, 6.58 and 20 pg per tube.

Specificity

Specificity of the two antisera used was determined by Abraham's method [21] (Table 1). Specificity of the method was also determined by overloading a plasma pool with 1 ng of steroid (Table 2).

Plasma values

Plasma concentrations of 3α- and 3β-diols were evaluated in 8 normal males (26–39 years old) and 7 normal females (25–34 years old). The concentrations (pg/ml + SD) of the two diols were respectively:
216 + 51 [172–318] and 49 ± 32 [9–104] for 3α-diol
239 ± 76 [139–401] and 82 ± 45 [17–137] for 3β-diol

DISCUSSION

Previous values reported for the level of 3α- and 3β-diols in human plasma are summarised in Table 3. For 3β-diol determinations, our results are significantly lower than the other published results. This fact may be explained by the purification step on celite microcolumns, allowing resolution of 5-androstene-3β,17β-diol and 3β-diol. This purification step was necessary because of the cross reactions of the antiserum with 5-androstene-3β,17β-diol (37%). In the method described by Toscano *et al.* [10] the purification step did not allow the separation between 3β-diol and 5-androstene-3β,17β-diol but the specificity of the antiserum used for 3β-diol determination (anti-DHT-1α-CETE:BSA) was very high (Cr%:0.5 for 5-androstene-3β,17β-diol). Nevertheless, the 3β-diol concentrations found in normal

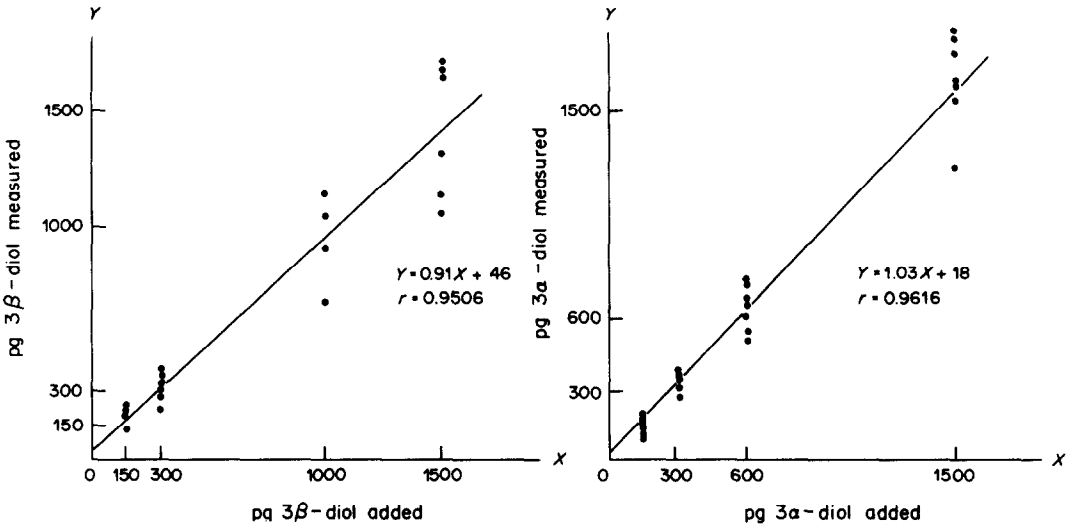

Fig. 3. Accuracy of method for radioiummunoassay of 5α-androstane-3β,17β-diol (3β-diol) and 5α-androstane-3α,17β-diol (3α-diol). Correlation curves between pg added and pg measured on a same plasma pool.

males and females are significantly higher relative to our results. It seems that a high specificity of the antiserum is necessary but does not eliminate the need for a purification step.

The antiserum for the 3α-diol assay cross reacted with 5-androstene-3α,17β-diol (21.6%) and the purification step on celite microcolumn did not separate 3α-diol from 5-androstene-3α,17β-diol (Fig. 1). Although this compound has not yet been measured in plasma, the following experience was carried out: a comparative assay of 3 plasma pools with and without a purification by HPLC which

Table 2. Interference due to 1 ng of steroid added to a plasma pool

Overloading	3α-Diol $\bar{X} \pm$ SD pg/ml	3β-Diol $\bar{X} \pm$ SD pg/ml Pool 1	3β-Diol $\bar{X} \pm$ SD pg/ml Pool 2
Plasma pool	131 ± 27	176 ± 43	424 ± 29
+ 5-Androstene-3α,17β-diol 1 ng	228 ± 20	—	—
+ 5-Androstene-3β,17β-diol 1 ng	—	—	448 ± 42
+ 5α-A-3α,17β-diol 1 ng	—	154 ± 40	—
+ 5α-A-3β,17β-diol 1 ng	117 ± 9	—	—
+ Androsterone 1 ng	130 ± 14	—	—
+ Epiandrosterone 1 ng	—	186 ± 23	—

Table 3. Determination of 3α-diol and 3β-diol in human plasma by different authors

Authors	3α-Diol mean ± SD pg/ml Males	3α-Diol mean ± SD pg/ml Females	3β-Diol mean ± SD pg/ml Males	3β-Diol mean ± SD pg/ml Females
Kinouchi and Horton [3]	137 ± 39	20 ± 6	—	—
Klemm *et al.* [4]	121 ± 24	—	—	
Barberial *et al.* [5]	152 ± 50	26 ± 5	—	—
Laband *et al.* [6]	189 ± 59	26.5 ± 2.7	—	—
Habrioux *et al.* [7]	267 ± 67	114 ± 33	816 ± 76	515 ± 177
Tamm and Volkwein [8]	251 ± 64	90 ± 20	426 ± 124	142 ± 29
Ghanadian and Puah [9]*	234 ± 7	—	—	—
Toscano *et al.* [10]	287 ± 86	108 ± 30	549 ± 290	239 ± 66
Hopkinson *et al.* [11]	—	—	607 ± 319	285 ± 67
Our results	216 ± 51	49 ± 32	239 ± 76	82 ± 45

*For [9]: mean ± SEM.

eliminated 5-androstene-3α,17β-diol from the plasma samples. The use of HPLC did not result in a great difference in the 3α-diol concentrations of the plasma pool after celite chromatography (100 ± 7 pg/ml) on the one hand and celite plus HPLC purification step (118 ± 19 pg/ml) on the other hand. The assay of the same plasma pool overloaded with 1 ng of 5-androstene-3α,17β-diol (for 3 ml of plasma) showed an increase of 81 pg with regard to the reference plasma (181 ± 9 pg/ml and 100 ± 7 pg/ml). Since cross reaction of the antiserum against 5-androstene-3α,17β-diol was about 21.6%, the calculated contribution of this interference to 3α-diol determination was 72 pg, against 81 pg measured, which shows a good agreement between the two values. After HPLC purification, the 3α-diol level decreased to 84 ± 7 pg/ml, a value near the base value. Thus the use of an HPLC purification step prevents interference due to the presence of 5-androstene-3α,17β-diol in human plasma. But this compound has not been identified in human plasma. In the future, the use of HPLC purification will guarantee the specificity of the method, with the elimination of 5-androstene-3α,17β and 3β,17β-diol from plasma samples.

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