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MEASUREMENT OF 5α-ANDROSTANE-3α.17β-DIOL AND 5β-ANDROSTANE-3α.17β-DIOL IN THE URINE OF HEALTHY MEN AND WOMEN

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SUMMARY

An assay for the determination in urine of 5α- and 5β-androstane-3α.17β-diol by gas–liquid chromatography (GLC) is described. Excretion rate of these urinary testosterone metabolites was calculated in healthy subjects (*5α-androstane-3α.17β-diol:* male: 141 ± 61 μg/24 h; female: follicular phase. 37 ± 17 μg/24 h. luteal phase. 31 ± 13 μg/24 h. *5β-androstane-3α.17β-diol:* male: 552 ± 312 μg/24 h; female: follicular phase. 181 ± 101 μg/24 h. luteal phase. 126 ± 84 μg/24 h.). These results suggest that the rate of 5α-androstane-3α.17β-diol excretion is sex dependent.

INTRODUCTION

BAULIEU and Mauvais-Jarvis[1, 2] have demonstrated the *in vivo* reduction of testosterone to 5α-androstane-3α.17β-diol and 5β-androstane-3α,17β-diol by pathways which do not involve oxidation to androstenedione. The reduction of testosterone occurs in two stages[3]: first. the double bond is reduced. producing either 17β-hydroxy-5α-androstan-3-one or 17β-hydroxy-5β-androstan-3-one. Then the keto function is hydrogenated predominantly to the 3α epimer. forming 5α-androstane-3α.17β-diol and 5β-androstane-3α.17β-diol.

The physiological and pathological importance of the "17β-hydroxyl" pathway[4–14] justifies the development of a method for measurement of these androstanediols in urine.

EXPERIMENTAL

Materials

Biological material. Urine was collected for 24 h over 10 mg sodium merthiolate (Merseptyl "Houdé").

Helix pomatia β-Glucuronidase (Industrie Biologique Française), 1000 I.U. per ml of urine.

Bacterial β-Glucuronidase (Sigma. 40.000 I.U. per g), 300 I.U. per ml of urine.

Reagents. Ethyl ether (Melle). Ether, 11, is treated with 10 ml of ferrous sulfate solution (60 g $FeSO_4$ + 6 ml H_2SO_4 + 110 ml H_2O). The aqueous phase is discarded and the organic phase washed with a saturated solution of potassium permanganate. Washing is continued until the permanganate color remains stable for at least 2 h. The ether is then adjusted to pH 7·0 with normal sodium hydroxide. After filtration through sodium sulfate. the ether is distilled twice in a glass apparatus with a 100 cm column. This solution is then distilled at 34·5°C. The distillate is collected and kept at 0°C in a colored glass flask.

Anhydrous sodium sulfate (Prolabo, 28111) was dehydrated in an oven at 800°C.

Anhydrous ethanol (Merck, reagent grade No. 6649). Ethanol is distilled after 6 h of refluxing over potassium hydroxide (3 g/l) and silver nitrate (2 g/l). The 78–79°C distillate is collected.

Benzene (Merck, reagent grade No. 1782). Benzene is washed with concentrated sulfuric acid until no color is present in the acid phase. Prior to distillation, the benzene is neutralized by washing with sodium hydroxide. Distillation is performed in a glass 150 cm column (reflux coefficient = 0·9). The 79·5–80°C distillate is collected. This distillate is kept in the cold and then saturated with distilled water in a colored glass bottle.

Alumina (Merck No. 1097, activity II. III according to Brockman[15]).

Hexamethyldisilazane (HMDS) from "Applied Science Laboratories" is purified under vacuum (30 mm Hg) with a 50°C nitrogen stream and kept in sealed tubes under anhydrous nitrogen.

Trimethylchlorosilane (TMCS) from "Applied Science Laboratories".

Tetrahydrofuran (Merck, reagent grade No. 9731). One liter tetrahydrofuran is refluxed for 6 h over 6 g ferrous sulfate. The fraction distilling at 65–68°C is collected over sodium and redistilled. The distillate collected at 66–66·5°C is kept cold in a colored glass bottle with molecular sieve.

Reagent for trimethylsilylation[16]. Hexamethyldisilazane–trimethylchlorosilane–tetrahydrofuran (10 : 1 : 89, by vol.). This reagent is stable for several weeks when kept dry in a glass stoppered flask.

Carbon disulfide (Merck, reagent grade No. 2214). One liter carbon disulfide is mixed with 100 g mercury and then distilled twice. The distillate collected at 46–47°C is kept at 0°C in a colored glass bottle with molecular sieve.

Gas chrom Q, "Applied Science Laboratories," 100–120 mesh.

Cyclohexane dimethanol succinate, "Applied Science Laboratories" (Hi-Eff 8 BP, or CHDMS).

Columns for chromatography 1·2 cm dia. × 25 cm, pyrex glass.

Tubes for thin layer chromatography (TLC) and GLC samples according to Scholler *et al.*[17].

GLC was performed on a Packard gas chromatograph of the 7800 series with a $\frac{1}{4}$ in. × 5 ft glass column, and on a Pye 104 gas chromatograph, model 84, with the same type of column; both of them were equipped with a flame ionization detector.

A standard procedure was used for column coating: 100 ml chloroform with 0·5% Hi-Eff 8 BP was added to 25 g Gas chrom Q 100–120 mesh and evaporated under slight vacuum and mild temperature. Packed columns were utilized after a 48 h conditioning at 220°C under nitrogen.

Steroids. 5α-androstane-3α.17β-diol was synthetized by potassium borohydride reduction of androsterone (Roussel UCLAF). The products of reaction were chromatographed on a gradient elution alumina column[18] using a donor mixture of 6% ethanol in benzene. Purity was checked by GLC and TLC. Identity was confirmed by infrared spectrometry.

5α-pregnane-3α.20α-diol (Merck No. 8955)

5β-pregnane-3α.20α-diol (Merck No. 8967)

5β-androstane-3α.17β-diol (Sigma No. 7880).

Methods

Collection and hydrolysis of urine. Twenty-four hour urine samples were collected over Merseptyl and kept at 0°C. Only a 5/100 aliquot of male urine or a 20/100 aliquot of female urine was hydrolyzed. This process was accomplished by incubating the urine specimens with Helix pomatia β-glucuronidase at 37°C for 12 h.

Extraction. After hydrolysis the free steroids were extracted once with an equal volume of ether and then twice with half the volume. The pooled ether extracts were washed twice with a tenth of their volume of normal sodium hydroxide and then washed twice with water in the same proportions. The washed ether extract was dehydrated on anhydrous sodium sulfate and dried down under vacuum.

Separation of hydroxysteroids with Girard's T reagent. Ten ml anhydrous ethanol, 1 ml acetic acid and 100 mg Girard's T reagent were added to the dried extract. This mixture was maintained at 37°C for 12 h. The pH was then adjusted to 6·4 with 12 N sodium hydroxide, and 10 ml of ice cold distilled water were added. The hydroxysteroids were extracted twice with 100 ml ice cold ether. The ether extract was washed with 10% sodium carbonate, then twice with 10 ml of distilled water, and dried under vacuum.

Fractionation on alumina column

(a) *Adsorption*

The dried extract was dissolved in 2 × 5 ml of water saturated benzene at 45°C. The benzene solution was adsorbed on an 8 g alumina column.

(b) *Elution*

Elution was performed with 10 ml benzene, 35 ml benzene–ethanol (99·2: 0·8, v/v). 40 ml benzene–ethanol (98:2, v/v). The last eluate, which contains the C_{19} and C_{21} dihydroxysteroids, was dried under vacuum at 40°C.

Purification by TLC. The dried extract was dissolved in methanol and transferred (2 ml + 1 ml) into a 10 ml conical tube. The solvent was dried and the residue dissolved in 200 μl of methanol. This mixture was then applied on a silica gel F_{254} thin layer plate. The tube was washed with 100 μl of methanol which was also applied on the thin layer plate. Standards (5 μg quantities) were applied adjacent on the thin layer plate to the urinary extracts. The plate was developed three times in CH_2Cl_2–ethyl acetate (9:1, v/v).

The standards zones were located by spraying with methanol–sulfuric acid (3:7. v/v) and heating at 120°C for 5 min. The position of revealed standards identified zones which corresponded to dihydroxysteroids present in the sample. These zones were then scraped off the plate. The gel was deactivated by three drops of water and eluted with 4 times 2 ml of a $CHCl_3$–methanol (2:1, v/v) mixture.

The eluates were collected and dried under nitrogen in a 10 ml conical tube.

Derivative formation. Both urinary extracts and standards were treated simultaneously; 1·5 ml of trimethylsilylation reagent mixture was added to each dried fraction, the tubes stoppered and left overnight at 37°C. The solvent was then evaporated at 60°C under a stream of nitrogen. The dry residue was finally dissolved in 200 μl CS_2.

GLC analysis

(a) *Settings*

The separation of trimethylsilyl–ether derivatives was performed on a Gas Chrom Q column with 2% cyclohexane dimethanol succinate (Hi-Eff 8 BP).

The following conditions were used:

oven temperature = 190°C

injector temperature = 230°C

detector temperature = 220°C

carrier gas = nitrogen. 60 ml/min. pressure = 2 bars

hydrogen to detector 30 ml/min. pressure = 1·5 bars

oxygen to detector 250 ml/min. pressure = 1·0 bar

flame ionization detector operated at a sensitivity range of 1×10^{-10} A.

(b) *Injection*

A 10 μl Hamilton syringe with Chaney adaptor was used; 2 μl of each urinary extract and 2 μl of each standard were injected into the gas chromatograph.

(c) *Evaluation of detector response*

Standard curves (produced by measuring the heights of the peaks for each standard compound) were obtained (Fig. 1) for the TMSi derivatives of:

5α-androstane-3α.17β-diol (1)

5β-androstane-3α.17β-diol (2)

5α-pregnane-3α.20α-diol (4)

5β-pregnane-3α.20α-diol (5).

The steroids present in the purified urinary extract were measured (Am) by comparing them to the corresponding standard curve.

The total quantity (Em) of each diol in the whole 24 h urine sample was determined by using the following formula:

$$Em = Am \cdot \frac{Q}{X}$$

where Q is the coefficient utilized to correct losses which occurred during the procedure. Q = radioactivity added to the urine sample/radioactivity in final sample; X = % of total urine sample which was processed; and Am = μg of injected steroids. determined from the standard curve.

RESULTS

The different TMSi derivatives of hydroxysteroids found in the purified urinary extracts are indicated in Table 1. Figures 2, 3 and 4 show chromatograms of standard mixture and urinary extracts from normal males and females.

The urinary values obtained for each identified hydroxysteroid in the different subjects studied are shown in Table 2.

The mean values for normal subjects are reported in Table 3: males excrete from 71 to 338 μg/24 h of 5α-androstane-3α,17β-diol in urine; females 21–60 μg/24 h in the follicular phase and 14–64 μg/24 h in the luteal phase. The excretion of 5α-androstane-3α.17β-diol between men and women is significantly different.

Males excrete from 110 to 1300 μg/24 h of 5β-androstane-3α.17β-diol in urine. Females excrete 54–400 μg/24 h in the follicular phase and 62–307 μg/24 h in the luteal phase.

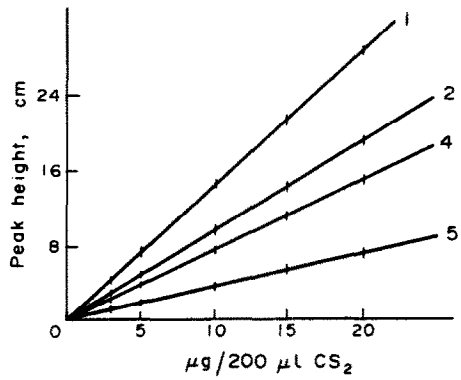


Fig. 1. Curves of standard steroids.
1 = 5α-androstane-3α.17β-diol
2 = 5β-androstane-3α.17β-diol
3 = 5-androstene-3β.17β-diol
4 = 5α-pregnane-3α.20α-diol
5 = 5β-pregnane-3α.20α-diol.

Table 1. Relative retention time of TMSi derivatives on Hi-Eff 8 BP stationary phase

TMSi derivatives	Relative retention time Our method	Other methods
1. 5α-androstane-3α.17β-diol	0·24	0·28[19]
2. 5β-androstane-3α.17β-diol	0·34	0·37[19]
3. 5-androstene-3β.17β-diol	0·42	
5α-androstane-3β.17β-diol	0·40	0·42[19]
4. 5α-pregnane-3α.20α-diol	0·56	0·55[20]
5. 5β-pregnane-3α.20α-diol	0·72	0·72[20]

Relative retention time is calculated on the basis of 5α-cholestane retention time, which was 50 min in our working conditions.

Nine months pregnant women excrete from 20 to 86 μg/24 h of 5α-androstane-3α.17β-diol and from 33 to 180 μg/24 h of 5β-androstane-3α.17β-diol.

DISCUSSION

Choice and elaboration of method

Among possible methods for the analysis of natural steroids, GLC was the most convenient in this laboratory. This technique allows analysis of isomers in micro amounts on appropriate stationary phases.

Free C_{19} and C_{21} dihydroxysteroids and their derivatives (trimethylsilyl-ether, trifluoroacetate, acetate, heptafluorobutyrate) have been tried on different stationary phases, either non polar (SE-30, OV-1) or polar (neopentyl-glycol-succinate, OV-17, XE-60, STAP, Hi-Eff 8BP)[21].

Three stationary phases were suitable for this work: neopentyl-glycol-succinate, XE-60 and Hi-Eff 8BP. We selected the last phase for two reasons: (1) the peak resolution of TMSi derivatives of 5α-androstane-3α.17β-diol and 5β-androstane-

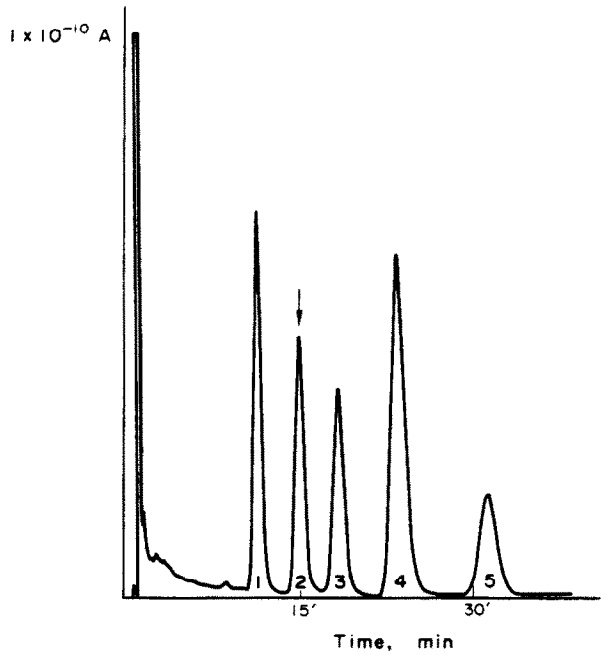


Fig. 2. Gas chromatographic separation of TMSi derivatives of the different standard steroids.

1 = 75ng 5α-androstane-3α,17β-diol
2 = 75ng 5β-androstane-3α,17β-diol
3 = 75ng 5-androstene-3β,17β-diol
4 = 150ng 5α-pregnane-3α,20α-diol
5 = 75ng 5β-pregnane-3α,20α-diol.

The retention time of 5-androstene-3β,17α-diol is shown by the arrow.

3α,17β-diol is superior (2), there is less interference from the 17α epimers.

Since GLC is a very sensitive technique, special care has to be taken through purification steps of the urinary extract.

Urinary hydroxylated metabolites of testosterone being glucuro-conjugated, β-glucuronidase was used but not acid hydrolysis which gives interfering products on GLC.

Very pure organic solvents must be used for extractions and for Girard's reaction and chromatography. To test their purity, TMSi derivatives of solvent residues collected after evaporation were injected on GLC as controls.

Utilizing the described method, we have been able to detect up to 5×10^{-9} g of 5α-androstane-3α,17β-diol with flame ionization detector. The linear response of this detector establishes a satisfactory standard curve. Reproducibility determined on 15 readings was satisfactory, the maximal deviation being 6%.

The losses caused by purification procedures were determined in urines of an immature female and normal men by means of added [1,2-^{3}H] 5α-androstane-3α, 17β-diol and non radioactive standards. The results are shown in Table 4. Immature female urine was used as a blank of the method because prepubertal plasma does not contain 17β-hydroxy-5α-androstan-3-one[22] which is a well known precursor for urinary 5α-androstanediols[3]. When processed in the

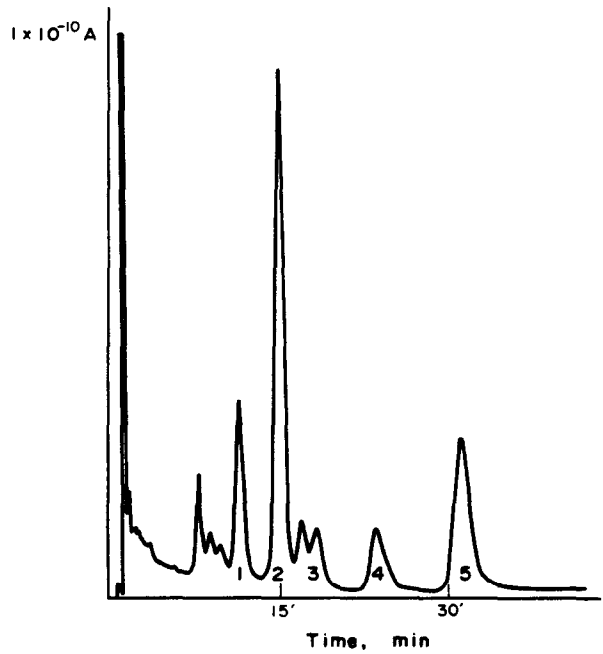


Fig. 3. Gas chromatogram of purified urinary extract from normal male.
1 = 5α-androstane-3α.17β-diol
2 = 5β-androstane-3α.17β-diol
3 = 5-androstene-3β.17β-diol
4 = 5α-pregnane-3α.20α-diol
5 = 5β-pregnane-3α.20α-diol.

described manner it was shown that the sample did not contain 5α-androstane-3α, 17β-diol or 5β-androstane-3α,17β-diol. The 5β-pregnane-3α,20α-diol (peak 5 of Fig. 5) was evaluated to 10 μg/24 h.

The benzene–ethanol 2% fraction from adsorption chromatography contains some androstanediol epimers capable of interfering in the measurement of the metabolites. TLC on silica gel F_{254} CH_2Cl_2-AcOEt 9:1, v/v, 3 successive developments) allows an excellent separation of 5α- and 5β-androstane-3α,17β-diol and 5β-androstane-3α.17α-diol (see Table 5). The 5β-androstane-3α.17α-diol has been isolated in urine [23]. This compound which could interfere on GLC is readily eliminated by TLC (Table 5).

By separate scraping and elution of the 5α-androstane-3α.17β-diol zone and of the 5β-androstane-3α,17β-diol zone, the exact amount of each diol can be determined by GLC.

In systematic investigations, it was possible to proceed with the simultaneous measurement of the two androstanediol metabolites after simultaneous scraping of the whole corresponding gel zone. In this case (see Table 5) only the 5-androstene-3β.17α-diol interferes in the measurement of 5β-androstane-3α,17β-diol. The contribution of this product isolated from urine [24] to the 5β-androstane-3α, 17β-diol peak has been studied on 14 normal subjects (11 males, 3 females) and was less than 12%. Consequently we think that physiologically the 5α- and the 5β-androstanediols can be measured simultaneously.

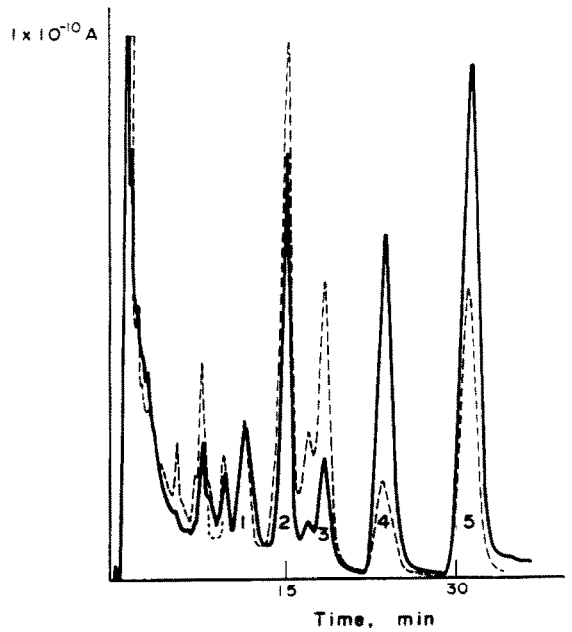


Fig. 4. Gas chromatogram of urinary extracts from normal women. Solid line: luteal phase. Dashed line: follicular phase. The peak 5 of luteal phase was at a sensitivity range of 2×10^{-10} A.

1 = 5α-androstane-3α.17β-diol
2 = 5β-androstane-3α.17β-diol
3 = 5-androstene-3β.17β-diol
4 = 5α-pregnane-3α.20α-diol
5 = 5β-pregnane-3α.20α-diol.

Specificity of the method is based upon:

High purification of urinary extracts.

Comparative study of steroid TMSi derivatives' retention times on Hi-Eff 8BP[21] (see Table 5). A GLC column giving an efficiency of 1500 theoretical plates per meter for 5α-cholestane is required for a good separation of different TMSi derivatives.

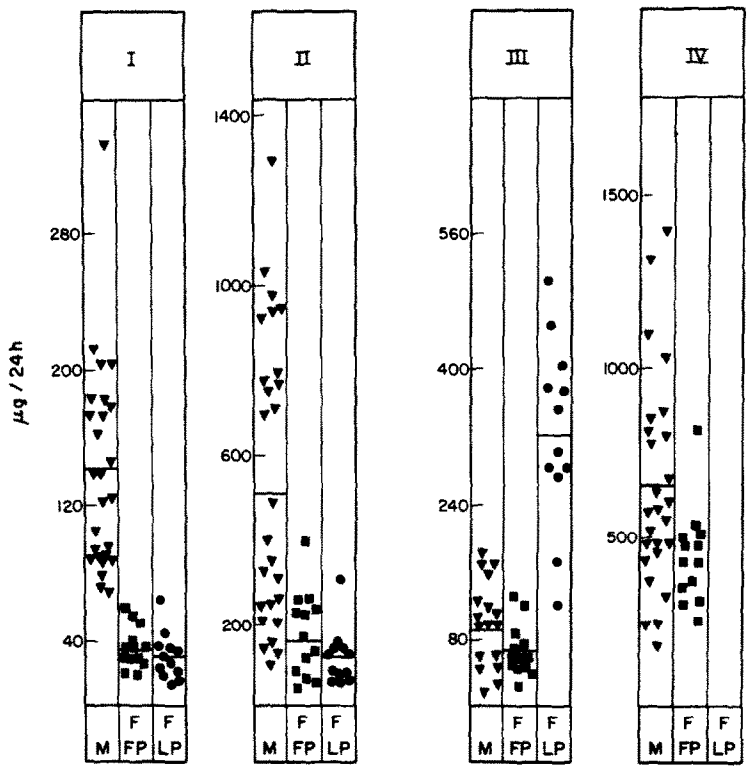
Blank obtained from urine of an immature female.

Identification of C_{19} dihydroxysteroids achieved by crystallization to constant specific activity. This method of sample purification was used to identify urinary 5α- and 5β-androstane-3α.17β-diol following injection of radioactive testosterone in normal subjects. These metabolites were finally crystallized to constant specific activity. Thus, an additional support to the purity of the final samples was obtained.

Association of GLC and TLC in this method. GLC and TLC deleted the possible interference of other steroids in androstanediol and pregnanediol peaks (Table 5).

Seven measurements determined on two stationary phases: XE-60 and Hi-Eff 8BP. The results are shown in Table 6. Thus, homogeneity of peaks corresponding to TMSi derivatives of 5α-androstane-3α.17β-diol and 5β-androstane-3α.17β-diol is shown.

Table 2. Individual quantitative determinations (μg/24h) of the different urinary steroids studied.
I = 5α-androstane-3α.17β-diol
II = 5β-androstane-3α.17β-diol
III = 5α-pregnane-3α.20α-diol
IV = 5β-pregnane-3α.20α-diol
F.F.P. = Follicular Phase Female
F.L.P. = Luteal Phase Female
M = Male



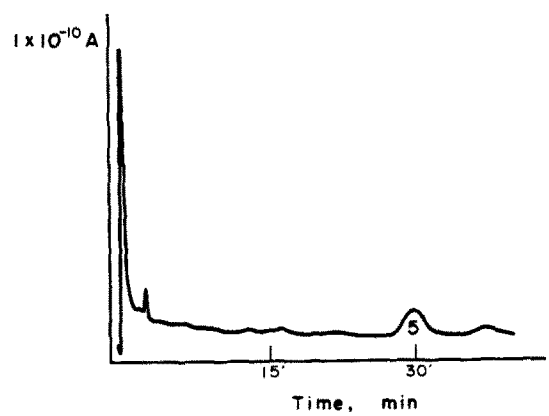


Fig. 5. Gas chromatogram of a purified urinary extract from half a 24 h sample of an immature female.
5 = 5β-pregnane-3α.20α-diol.

Table 3. Mean values and ranges (μg/24h) of the different urinary steroids studied

Sex		Number of cases	5α-androstane-3α,17β-diol (μg/24 h)		5β-androstane-3α,17β-diol (μg/24 h)		5α-pregnane-3α,20α-diol (μg/24 h)		5β-pregnane-3α,20α-diol* (μg/24 h)	
			Mean $\pm\sigma$	Range	Mean $\pm\sigma$	Range	Mean $\pm\sigma$	Range	Mean $\pm\sigma$	Range
M	Normal	26	141 ± 61	71–338	552 ± 312	110–1300	102 ± 52	15–185	650 ± 311	185–1400
F	Follicular phase (F.P.)	13	37 ± 17	21–60	181 ± 101	54–400	69 ± 31	43–138	443 ± 142	250–810
	Luteal phase (L.P.)	13	31 ± 13	14–64	126 ± 84	62–307	321 ± 108	120–500	Quantities are too large to be measured by this method	
	Pregnant 9th month (P)	4	49 ± 33	20–86	75 ± 70	33–180	510 ± 84	450–600		

σ = standard deviation calculated from the expression
X = value obtained from individual measurements

$$\sigma = \sqrt{\frac{\Sigma [X - \bar{X}]^2}{N - 1}}$$

$\overline{X}$ = mean of measurements
N = number of independent observations

*Mean value of 5β-pregnane-3α,20α-diol urinary excretion is in agreement with the one found by R. Scholler *et al.*[20] when utilizing gas–liquid chromatography.

Table 4. Recovery of steroids at different purification steps. The mean value obtained with 30 samples led to a factor $Q = 100/65$

Purification steps	Recoveries range	Recoveries mean
Extraction after hydrolysis	80–90%	89%
Extraction after Girard	80–90%	84%
Adsorption column	75–84%	80%
After TLC	63–75%	65 ± 5%

Table 5. Isomers of androstanediols and pregnanediols: separation by TLC and GLC combination

Steroids	Relative retention time of TMSi derivatives to retention time of 5α-cholestane	TLC R_T* in CH_2Cl_2-AcOEt (9 : 1, v/v), 3 migrations, silica gel F_{254}
5β-androstane-3β.17α-diol	0·18	0·82
5α-androstane-3α.17α-diol	0·21	0·42
5β-androstane-3α.17α-diol	0·22	0·22
5α-androstane-3α.17β-diol	0·25	1·00
5β-androstane-3β.17β-diol	0·25	1·56
5α-androstane-3β.17α-diol	0·32	0·96
5-androstene-3β.17α-diol	0·34	1·04
5β-androstane-3α.17β-diol	0·34	0·55
5α-androstane-3β.17β-diol	0·40	1·20
5-androstene-3β.17β-diol	0·42	1·26
5α-pregnane-3α.20β-diol	0·49	1·23
5β-pregnane-3β.20β-diol	0·50	1·20
5α-pregnane-3α.20α-diol	0·56	0·98
5β-pregnane-3β.20α-diol	0·57	1·48
5β-pregnane-3α.20β-diol	0·66	0·70
5β-pregnane-3α.20α-diol	0·72	0·55
5α-pregnane-3β.20β-diol	0·80	1·42
5α-pregnane-3β.20α-diol	0·88	1·18

*Running rate relative to 5α-androstane-3α.17β-diol.

Significance of results

Mauvais-Jarvis *et al.*[2, 5, 6] have shown testosterone and dehydroepiandrosterone to be precursors of urinary androstanediols. Therefore, the amount (A) of either 5α-androstane-3α.17β-diol or 5β-androstane-3α.17β-diol was calculated by using the following equation:

$$A = T \,.\, R_T + D \,.\, R_D$$

where T = daily testosterone production, D = daily dehydroepiandrosterone production, R_T = conversion rate of testosterone to one of the two urinary androstanediols. R_D = conversion rate of dehydroepiandrosterone to one of the two urinary androstanediols.

Utilizing data obtained by Mauvais-Jarvis and Baulieu [2] we calculated urinary 5α-androstane-3α.17β-diol to amount to 45–165 μg/24 h in male and 11–48

Table 6. Measurement of urinary androstanediols and pregnanediols on XE-60 and Hi Eff 8 BP stationary phases

Sex	5α-androstane-3α, 17β-diol (μg/24 h)		5β-androstane-3α, 17β-diol (μg/24 h)		5α-pregnane-3α, 20α-diol (μg/24 h)		5β-pregnane-3α, 20α-diol (μg/24 h)	
	XE-60	Hi-Eff 8 BP	XE-60	Hi-Eff 8 BP	XE-60	Hi-Eff 8 BP	XE-60	Hi-Eff 8 BP
M	140	140	920	980	95	99	480	472
M	166	166	680	708	15	15	180	185
M	347	338	900	955	195	185	615	615
M	129	123	780	770	61	64	380	384
F.FP	21	22	162	168	50	49	370	350
F.LP	14	14	85	76	184	176	> 2 mg	
F.LP	34	35	292	307	390	370	> 1·5 mg	

μg/24 h in female. Urinary 5β-androstane-3α.17β-diol amounted to 162–903 μg/24 h in male and 66–185 μg/24 h in female. These values are in agreement with those measured by GLC in duplicated aliquots of the 24 h urine sample (see Table 3).

In males the main part of 5α-androstanediol probably originates from the hepatic and also extrahepatic metabolism of testosterone. In females, 5α-androstanediol essentially arises from testosterone formed in the liver from the conversion of dehydroepiandrosterone and androstanedione [2–5].

The differences observed between males and females in the urinary excretion of 5β-androstanediol are mainly sex linked variations of the hepatic testosterone metabolism [5].

As long as 5α-androstanediol might be formed in part from the metabolism of testosterone in target tissues, especially the skin [13], its determination in urine is interesting and might be a better index of androgenicity than testosterone itself.

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