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STEROID PROFILES OF HEALTHY INDIVIDUALS

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SUMMARY

Urine steroid profiles of healthy individuals can be divided into two groups according to greatly different excretion rates of dehydroepiandrosterone (DHEA). About 80% of the population show an excretion of DHEA in urine of just above the detection limit or less of the main androgens androsterone (A) and etiocholanolone (E). This excretion is only enhanced in psychological stress situations. The remaining 20% excrete DHEA in roughly equal amounts as A and E.

While the relation of excreted steroids is rather constant, the absolute amounts may vary greatly. In contrast to the behaviour of all other steroids DHEA excretion is not in relation to other steroids. The group of "high DHEA" producing individuals in particular shows drastic changes in the excretion during a day: the DHEA excretion rapidly rises from morning until afternoon and then drops to rather low values in the resting period during the night. A recognizable DHEA production seems to be closely related to the waking period.

INTRODUCTION

Steroid profiles [1,2] obtained by a gas chromatographic separation of the steroid fraction from body fluids — after appropriate derivatisation to enhance volatility and thermal stability — allow an insight into steroid metabolism. In the course of investigation of the urine steroid profiles of hirsute women we observed in many cases a great (10—30-fold) increase of the amount of dehydroepiandrosterone (DHEA) excreted compared with the excretion of normal individuals [3]. A further study revealed that psychological stress situations stimulate DHEA production by one order of magnitude or more [4]. In the widespread literature, mainly related to 17-ketosteroids, very different and sometimes contradictory reports on DHEA excretion are found [5—7].

"Normal" profiles published in literature show very different DHEA excretions, in most cases not mentioned explicitly. All these results initiated a detailed study of the excretion of steroids in our laboratory — especially of

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DHEA. Samples were taken in 4-h intervals lasting for 3 days to one month to obtain a detailed insight into individual changes in steroid excretion during these periods in order to be able to detect the reasons for the differences in DHEA excretion and to be able to recognize abnormal and possibly pathological changes more easily.

MATERIALS AND METHODS

Collection of samples

Urine samples were ccllected from healthy individuals 20—40 years' old. During the collection period lasting between 3 and 28 days the individuals lived on normal diet (usually "mensa" food). Coffee drinking was not allowed since this causes interference to the profiles by peaks arising from cauran acids occurring in coffee beans [8]. Samples were collected every 4 h and stored at —18°C. Although only small (10 ml) samples were used for analysis, the whole daily urine production was collected in order to quantify the daily rates of excretion products.

Work-up procedure

The neutral steroids were obtained according to a somewhat modified procedure introduced by Horning et al. [9]. Samples showing high DHEA levels were subjected, after the enzymatic hydrolysis, to solvolysis to cleave the sulfates — especially those of DHEA — completely. The values of DHEA obtained by this procedure are 35—40% higher compared to those obtained by enzymatic hydrolysis alone.

To reduce the danger of unexpected additional peaks interfering with a single added standard compound, we used various standards: eicosanol, hexadecanediol and epi-etiocholanolone [10]. If the standards were not in the correct relation, the composition of the sample was checked by gas chromatography—mass spectrometry (GC—MS) for by-products hidden under the peak of a standard compound.

To a 10-ml aliquot of urine 20 μg of each internal standard compound (see above) were added.

Preparation of derivatives

In general, two methods of derivatisation are used: either trimethylsilylmethoxime (TMS-MO) derivatives [11] are prepared or the steroids are transformed into enol-trimethylsilyl (enol-TMS) ethers [12]. We prefer the preparation of enol-TMS ethers, since they can be prepared in one step and elute from the column much earlier than the corresponding MO-TMS derivatives. The disadvantage that the reaction is sometimes incomplete can be overcome by heating the samples for another couple of hours if incomplete reaction is observed.

Both derivatisation methods afford reproducible and exact values for most of the steroids. Only the determination of 11-oxygenated steroids remains problematic. The 11-oxygen function is rather sensitive to absorption, so that (depending on the column) sometimes great losses must be taken into account. This problem has been studied in this laboratory in detail, since changes in

11-oxygenated steroid concentrations seem to be of diagnostic value [13].

The residue obtained by working up 10 ml of urine was dissolved in 2 ml of methanol. Two-fifths of this solution were transferred to a reaction vessel (1 ml) with a Teflon screw cap and brought to dryness in vacuum at 40—50°C. Five microlitres of pyridine (free of water), 5 μl of tetrahydrofuran and 20 μl of N-trimethylsilyl-N-methyl-trifluoroacetamide (MSTFA) were added; 10 μl of the clear solution were transferred to a small glass tube (ca. 1 mm I.D., ca. 7.5 cm long) containing a crystal of anhydrous sodium acetate. The contents of the tube were air-tight melted and heated to 70°C for 5 h. After cooling 0.5—1.0 μl of the solution was analyzed by GC.

Assignment of structures

The correctness of structure assignment in the gas chromatograms was confirmed using a GC—MS—computer combination.

Calculation of excretion rates

Since it was very difficult in practice to obtain urine samples collected in an exact 4-h rhythm, the excretion rates were calculated on the basis of μg/h for each collection time unit (about 2—4 h).

Usually quantification relies on the peak area of added epi-etiocholanolone (epi-E) [10]. The use of this internal standard requires the use of glass capillary columns of high quality to avoid interference with the peaks of androsterone (A) which elute directly after epi-E in the chromatogram (Δ RI = 8—9).

The calculation of excretion rates of the steroid metabolites is based on the following formula [14]:

$$m_{\text{steroid}} = \frac{\text{peak area}_{\text{steroid}} \times n\, V_{\Delta t}}{\text{peak area}_{\text{I.S.}} \times f\, V_{\text{urine aliquot}} \times \Delta t}$$

where m_{steroid} = excretion rate of the steroid to be calculated (in μg/h), f = GC response factor for the steroid to be calculated, n = mass of internal standard (I.S.) added (in μg), $V_{\Delta t}$ = volume (in ml) of urine collected during Δt hours, Δt = period of time (in h) during which the urine was collected.

INSTRUMENTS AND CHEMICALS

Gas chromatography

Gas chromatograms were taken on a Siemens-L 402 gas chromatograph equipped with a flame-ionization detector. The column was a 25-m open tubular glass capillary (0.3 mm I.D.) wall-coated with OV-101 [15]. Hydrogen carrier gas pressure was 0.8 bar. Temperature program was 2°C/min from 150 to 300°C. Detector temperature was 280°C while the injection port was kept at 260°C. Split ratio was 1:10. Peak area integration was performed by an Autolab System 1 (Spectra-Physics) computing integrator.

Kováts retention indices were found using a standard mixture of even carbon number hydrocarbons from C-16 to C-34.

Gas chromatography—mass spectrometry

Mass spectra were obtained with an LKB 2091 instrument. The electron ion

TABLE I

IDENTIFICATION OF GC PEAKS IN URINARY STEROID PROFILES OF FIGS. 1--4, 7

GC peak No.	Systematic name	Retention index (OV-101)	Molecular weight	Mass-spectra ions [*m/e* (%)]
1	3α-Hydroxy-5α-androst-16-ene, TMS	2186	346	75(68), 94(68), 131(29), 148(42), 241(100), 256(36), 331(15), 346(24)
St 1	1,16-Dihydroxy-hexadecane (hexadecanediol); di-TMS: internal standard	2321	402	73(85), 75(100), 83(90), 97(68), 103(52), 147(74), 149(70), 297(5)
St 2	Eicosanol, TMS: internal standard	2360	370	69(20), 73(34), 75(76), 83(20), 97(18), 103(27), 111(8), 355(100)
St 3	Enol ether of 3β-hydroxy-5β-androstan-17-one (epi-etiocholanolone); di-TMS: internal standard	2519	434	73(100), 143(36), 169(40), 182(22), 239(20), 329(43), 344(13), 419(90), 434(80)
4	Enol ether of 3α-hydroxy-5α-androstan-17-one (androsterone), di-TMS	2527	434	73(100), 143(36), 169(40), 182(22), 239(20), 329(43), 344(13), 419(90), 434(80)
6	Enol ether of 3α-hydroxy-5β-androstan-17-one (etiocholanolone), di-TMS	2534	434	73(100), 143(36), 169(40), 182(22), 239(20), 329(43), 344(13), 419(90), 434(80)
7	3α,17β-Dihydroxy-5α-androstane, di-TMS	2546	436	73(100), 129(55), 215(32), 241(46), 256(54), 331(9), 346(23), 421(5), 436(7)
8	Enol ether of 3β-hydroxy-androst-5-en-17-one (dehydroepiandrosterone), di-TMS	2600	432	129(18), 147(15), 169(10), 303(8), 327(11), 342(3), 417(18), 432(23)
9	3β,17β-Dihydroxy-5-androstene, di-TMS	2620	434	129(90), 213(35), 215(56), 239(44), 254(40), 305(38), 329(25), 344(50)
10	Enol ether of 3α-hydroxy-5β-androstane-11,17-dione (11-keto-etiocholanolone), di-TMS	2640	448	168(21), 182(10), 304(53), 343(68), 358(50), 433(12), 448(22)

droitinase AC (10 units/ml). The exhaustive digestion was carried out at 37°C for 5 h. Standard chondroitin sulfates were also digested in the same manner to check the enzyme activity. Four volumes of absolute ethanol were added after incubations and the mixture left overnight at 4°C. The clear supernatant obtained by centrifugation was dried under a stream of nitrogen and the residue, dissolved in 90% methanol, was applied to the chromatograph for analysis.

Recovery of added isomeric chondroitin sulfates

Solutions were prepared which contained 1 mg/ml of standard C-4S, C-6S and DS in distilled water. Aliquots of 50 μl of each of these solutions were added separately to 2 ml of normal urine. The GAG in these augmented urines were precipitated with 100 μl of 5% CPC in 0.9% sodium chloride and isolated as described above. Reproducibility of recovery was measured by duplicate isolations of GAG of each spiked normal urine sample. Enzyme reaction conditions and sample treatment for HPLC analysis were the same as described above.

HPLC

In the HPLC separations, the eluent used was acetonitrile—methanol—ammonium formate buffer. A Whatman Partisil-10 PAC, a bonded cyano-amino-type column was used at a flow-rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Ratio of acetonitrile to methanol concentration in the eluent

The capacity ratios (k') of the disaccharides (ΔDi-0S, ΔDi-6S and ΔDi-4S) were determined as a function of the acetonitrile and methanol content of the mobile phase, while keeping the content of ammonium formate buffer constant at 20% (Fig. 1). Benzene was used as the inert peak in the calculations of the k' values. For sulfated disaccharides the k' values first decrease with increasing acetonitrile content and decreasing methanol content, pass through a minimum at an acetonitrile—methanol ratio of about 3.0 and then increase sharply. The k' value of nonsulfated disaccharide increases slightly in the region in which k' values of sulfated disaccharides decrease and then increases sharply as did the sulfated disaccharides. Fig. 1 demonstrates that the acetonitrile and methanol content is a valuable parameter for adjusting the retention. It is found that at a ratio of acetonitrile to methanol of 3.0, effective separation and good resolution of the three disaccharide isomers in the enzyme digest of urinary GAG is achieved. Thus, in further investigations of the influence of parameters in this aqueous buffer, this ratio for the two organic solvents was used.

Effect of pH, concentration and content of aqueous ammonium formate

The dependence of the k' values on the pH of ammonium formate buffer for the three unsaturated disaccharides was studied. For sulfated disaccharides the k' values decrease with an increasing pH of the buffer. However, the k' value of ΔDi-0S only changes slightly when the pH was varied from 3.5 to 5.75. Peak shapes of the three compounds become more symmetrical as the pH is

source temperature was 250°C, the electron energy 70 eV. The total ion current signal was registered at 20 eV. The GC column and temperature program were identical to those given above.

Chemicals

The chemicals used (and their source) were as follows: hexadecanediol (Aldrich, Milwaukee, WI, U.S.A.); 3β-hydroxy-5β-androstan-17-one (E. Merck, Darmstadt, G.F.R.); MSTFA (Macherey, Nagel & Co., Düren, G.F.R.); helicase (Boehringer, Mannheim, G.F.R.).

RESULTS AND DISCUSSION

Samples were taken from 15 healthy female and 30 male subjects. From every subject 18—20 urine samples were collected in 4-h intervals. To confirm the results, the collection was repeated after a six-week interval.

Urine steroid profiles of healthy individuals are characterized by two main peaks in the region of the androgens — androsterone (A) and etiocholanolone (E) — and one main peak in the region of the corticoids — tetrahydrocortisone (THE) (Fig. 1). Usually these three peaks are of comparable size. A much lower intensity of the peak corresponding to THE is a strong indication that the derivatisation was not properly done and corticosteroids were lost to a large extent [4,10].

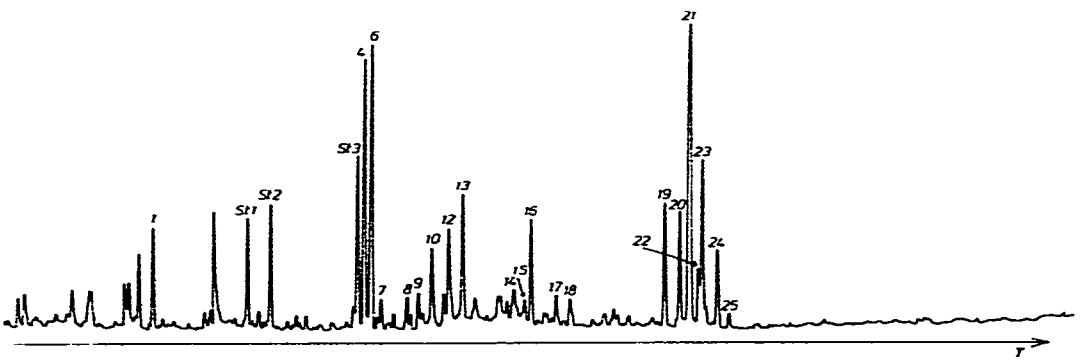

Fig. 1. Urinary steroid profile (TMS-enol-TMS ether) of a healthy 30-year-old male; steroids are identified in Table I.

Earlier it was reported [16] that male subjects always show a much higher excretion of A than of E. Although this is true in many cases, we found several male subjects with an equal or even higher ratio of E/A. An unambiguous distinction between the sexes by an inspection of these ratios therefore seems not to be possible.

The ratio between A and E differs from individual to individual, but the A/E ratio of urine samples of a single individual taken at different times does not differ greatly (Fig. 2).

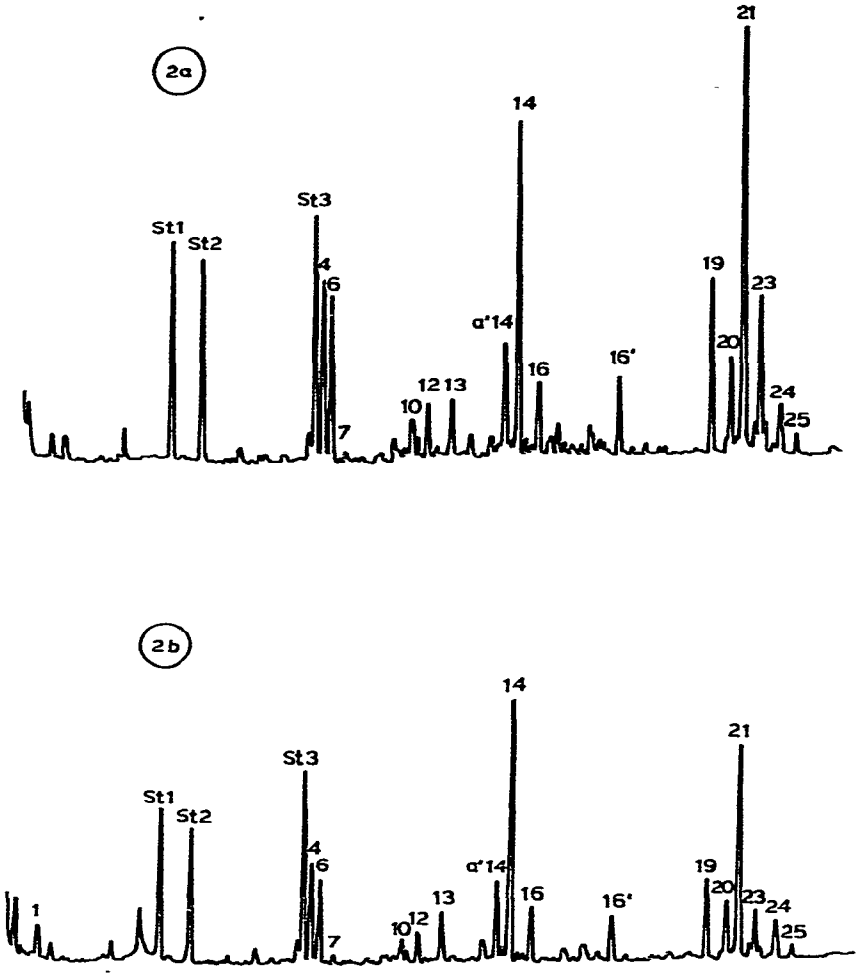

Fig. 2. Comparison of urinary steroid profiles (TMS-enol-TMS ether) of a female (30 years' old, luteal phase of cycle), taken from a 24-h urine specimen (2a) and at 1700 on the same day (2b); steroids are identified in Table I.

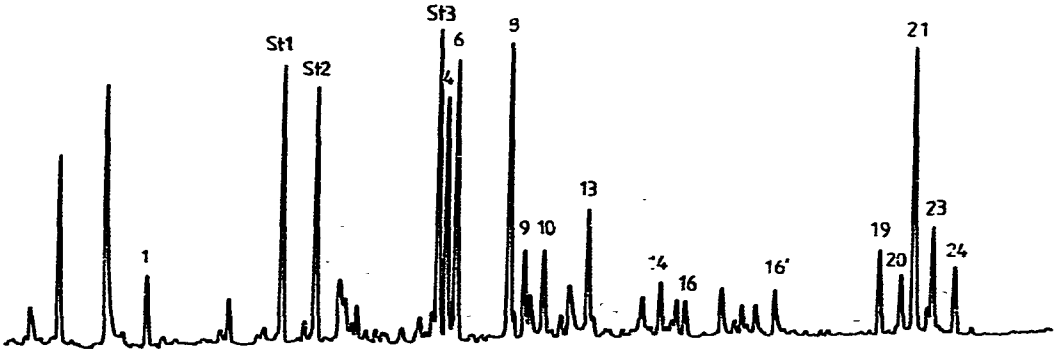

Fig. 3. Urinary steroid profile (TMS-enol-TMS ether) of a "high" DHEA excreting female (25 years' old); steroids are identified in Table I.

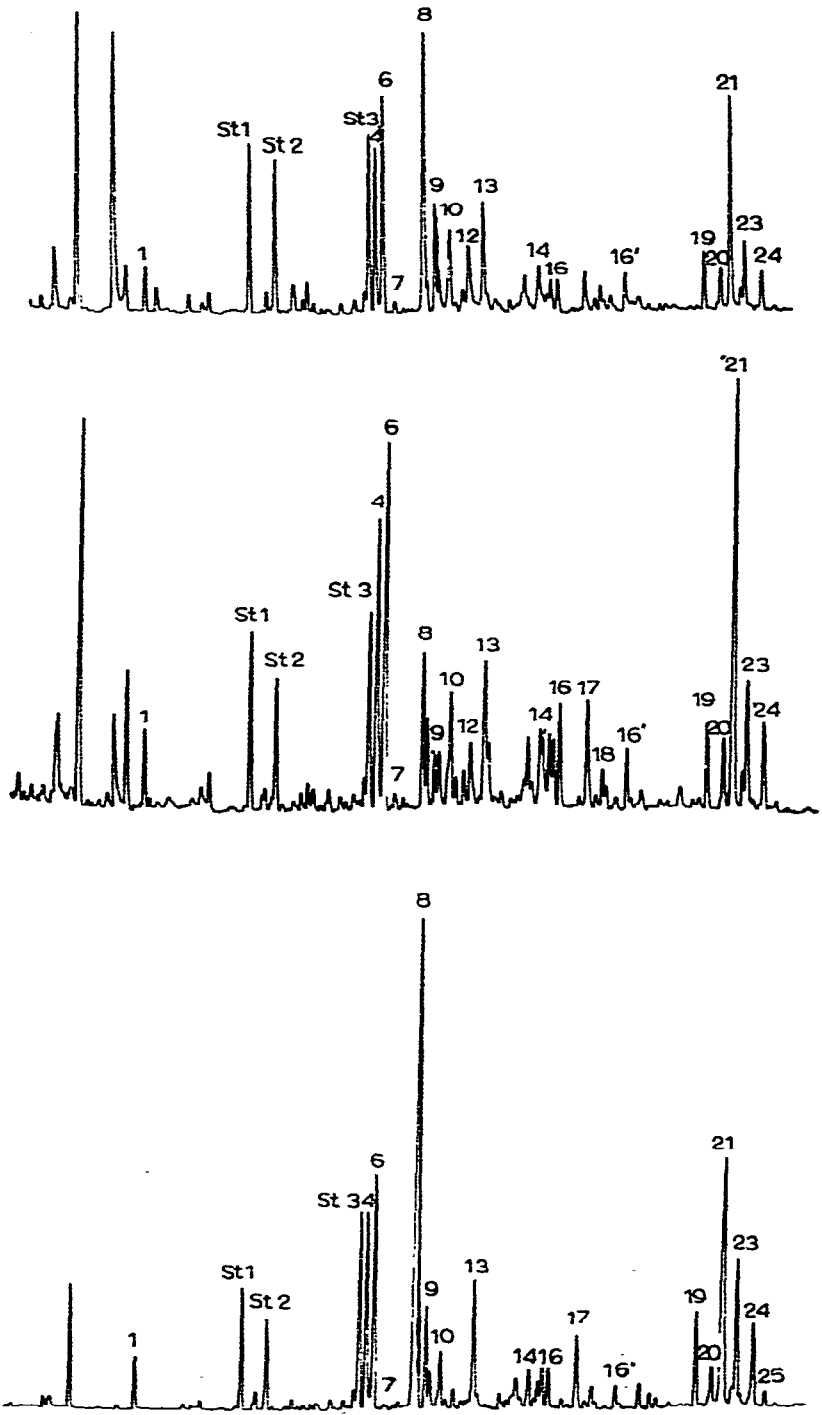

Fig. 4. Urinary steroid profiles (TMS-enol-TMS ether) of a "high" DHEA excreting female (25 years' old) taken from urine samples at different times of day: 2200 (top profile), 0600 (centre profile), 1000 (bottom profile); steroids are identified in Table I.

While the relative excretion of nearly all steroids varies in healthy individuals within certain limits (about 1:5), that of DHEA is subject to large changes (1:50). Usually the excretion of DHEA in a normal individual is just above the detection limit. Only in psychological stress situations is its excretion enhanced in most individuals to amounts comparable to those of A and E, i.e. 10—50-fold.

In this study about every tenth individual showed an excretion rate of DHEA in the same ratio as normal individuals do in a severe psychological stress situation (Fig. 3).

The excretion of DHEA in these persons changes a lot during one day, reaching a maximum about noon or in the afternoon and being low in the resting period (Fig. 4). Even in the resting period the DHEA excretion is about ten times higher than in a "normal" person. To exemplify this the ratio between DHEA and E of a "normal" DHEA excreting person and that of a high DHEA excreting person were compared (Table II).

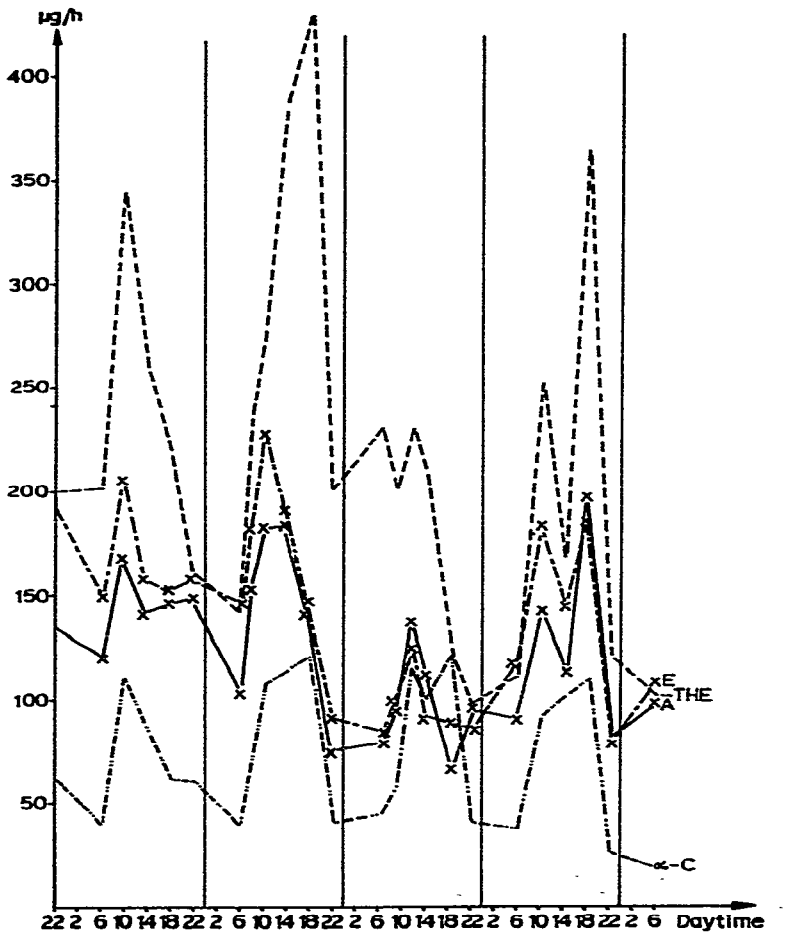

Fig. 5. Comparison of the excretion rates of androsterone (A), etiocholanolone (E), tetrahydrocortisone (THE) and ∝-cortolone (∝-C) of a male (28 years' old) over a 4-day collection period; values expressed in µg/h per collection time unit.

TABLE II

A/E AND DHEA/E RATIOS OF A "NORMAL" DHEA EXCRETING PERSON (I) COMPARED WITH A "HIGH" DHEA EXCRETING PERSON (II) FROM URINE SAMPLES COLLECTED IN A 3—4-h RHYTHM

	2200—0600		0600—1000		1000—1300		1300—1700		1700—2200	
	I	II	I	II	I	II	I	II	I	II
A/E	0.97	0.63	1.09	0.98	1.12	0.89	1.00	0.77	0.91	0.74
DHEA/E	N.D.*	0.36	0.04	3.10	0.10	3.13	0.24	2.22	N.D.*	0.82

*Not detectable.

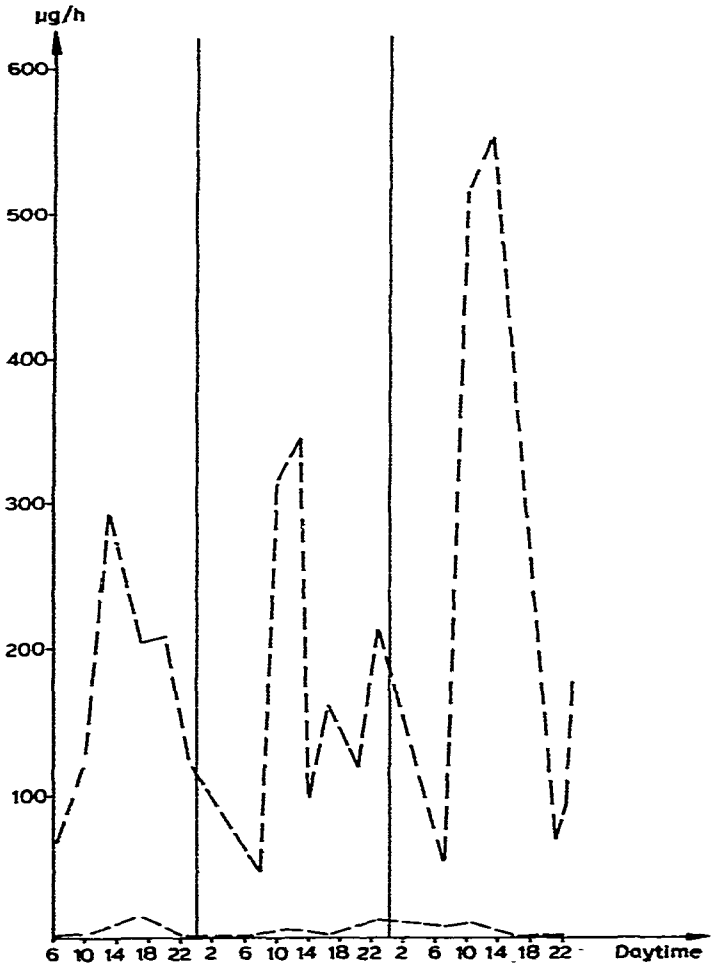

Fig. 6. Drastic differences in DHEA excretion rates between a female (25 years' old) typical of a "high" DHEA excreting person, and a female (30 years' old) typical for a "normal" DHEA excreting person.

It is therefore necessary to distinguish between two types of individuals: those who are living with a low excretion level of DHEA (about 80—90%) and those with a high excretion level of DHEA. This finding explains the great differences in the DHEA levels published [17].

Interestingly, high DHEA excretion is very common in women suffering from idiopathic hirsutism. About 60% of them are high DHEA excretors, a much higher percentage compared to other individuals. This is probably not due to altered adrenal enzyme activity, since there is no evidence for an abnormality of 3β-hydroxysteroid dehydrogenase in any of the hirsute subjects examined [18].

So far we only compared relative rates directly deducible from the profiles. The addition of an internal standard and knowledge of the volume of urine produced within a certain period enable us to calculate the quantitative steroid excretion at different times.

Although the relative rates of steroids excreted are rather constant (Fig. 5) the actual excretion rates change a good deal during the daytime, reaching a maximum about noon (Fig. 5). This effect, well known from group determinations [19—21], can be masked in clinical analysis by taking 24-h specimens.

Moreover, the excretion of urinary steroids also changes from day to day. These changes are not considered in clinical analysis although they are of great importance. Especially striking is the change in the daily excretion of DHEA in "high" DHEA excreting individuals compared to the low excreting individ-

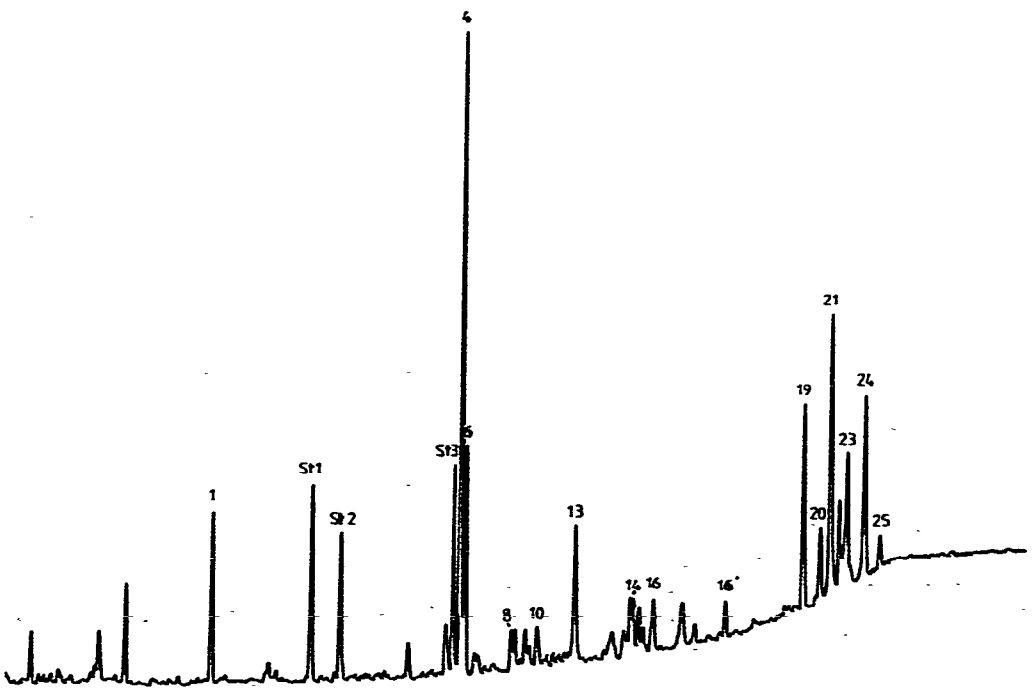

Fig. 7. Urinary steroid profile (TMS-enol-TMS ether) of a male (25 years' old) showing an extremely high A/E ratio; steroids are identified in Table I.

uals (Fig. 6), which is evident even by inspecting the profiles (Fig. 4) without reference to the standard.

One additional observation deserves to be mentioned. The profiles of 29 male subjects showed an A/E ratio of 2:1 or less; only one male subject developed a profile with a 3:1 ratio (Fig. 7). A similarly extreme ratio of the corticoids α-THF/THF > 1 was observed. Quantification revealed a normal E excretion but an increase in A excretion by a factor of 3—4 and in 3α-hydroxy-5α-androst-16-ene by a factor of similar magnitude. This individual obviously has a much higher activity of 5α-reductase than usual. Shackleton et al. [22] described a case of hypertension in which an extreme activity of this enzyme was found.

It must be assumed that there exist even more types of people distinguishable according to their metabolism. It would be of interest to investigate if these subjects also show an alternative rate of drug metabolism.

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