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ISOLATION OF 1,4-ANDROSTADIENE-3,17-DIONE FROM URINE OF AN EPILEPTIC BOY

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SUMMARY

We report the isolation and characterization of a steroid, 1,4-androstadiene-3,17-dione from the urine of a 15-yr-old boy with a history of epileptic convulsions and premature puberty. To our knowledge, this is the first report of detection of this steroid in human urine. A substance from the urine of an epileptic boy, under prolonged treatment with diphenylhydantoin and phenobarbital, was characterized and shown to be 1,4-androstadien-3,17-dione. This characterization depended preliminarily on examining the behaviour of the substance and its derivatives on thin-layer, paper and gas chromatographic systems, and conclusively by physico-chemical analysis of its spectra in U.V., I.R., circular dichroism, nuclear magnetic resonance, and gas chromatogram–mass spectrometer. The question of whether the appearance of this substance in urine was caused by the therapeutic handling or whether this steroid is a true intermediate during aromatization of testosterone and androstenedione is discussed.

SUBJECT

The patient, J.R., was a 15-yr-old boy when first seen by one of us (J.R.B.) and had a height of 158·5 cm and weight of 50·7 kg. His pubic hairs were those of a stage 5 boy [1] corresponding to a chronological age of 17 yr but without any abnormal sexual behaviour. His skeletal maturation was advanced for his age.

The subject was born micro-cephalic and the birth was of long duration without documentation of any injuries. He was cross-eyed and had an operation at an early stage of his life. At the age of 7 yr he started having epileptic convulsions and at the age of 14 his penis was markedly enlarged. His left testes grew larger than normal and a hard mass could be felt at its upper end.

His verbal I.Q. was 52 and practical I.Q. was too low to be measured. His E.E.G. showed a pathological pattern, without any differentiation of the waves, indicating convulsion potentials. The basic activity of the rhythm was found to be too low. At the age of 15, the patient underwent exploratory surgery of the left testis and was hydrocelectomized. The testicular biopsy demonstrated normal size tubules, and the picture was consistent with that of the last stage of pubertal development.

Steroid assays (average of four estimations) in the urine and blood was as follows, urinary 17-oxosteroids, 9·62 mg/day (androsterone 3·60 mg, aetiocholanolone—2·20 mg and dehydroepiandrosterone—2·15 mg) 17-hydroxycorticosteroids, 0·82 mg/day (cortisol metabolites—0·44 mg), testosterone, 53 μg/day and epitestosterone 30 μg/day. Plasma testosterone was 476 ng/100 ml, and urinary production rate of testosterone glucuronide 4·3 mg/day. Following ACTH administration plasma cortisol was raised from a control value of 8 μg to 19 μg/100 ml, a lower than normal response. The surgical procedures did not influence the steroid values and only a transient depression in the 17-oxosteroids was noted. When compared to the normal values [2, 3] these results indicate that our patient had higher urinary testosterone, epitestosterone and DHA, plasma testosterone and urinary production rate of testosterone. The 17-hydroxycorticosteroids, on the other hand, were extremely low.

The subject was given daily 1 g of 5-carbamoyl-5-H-dibenzoazepin, 250 mg of diphenylhydantoin and 0·1 mg of acid phenylethyl barbiturate, and during steroid studies no therapy was withdrawn.

METHODS AND RESULTS

The unknown steroid was first detected on a gas chromatogram as a distinct peak with a retention time of 34 min (under similar conditions androsterone had an r.t. of 5·5 min) well separated from the usual urinary steroids, when the TMSE derivative of the sample from Fraction II of the paper chromatogram [5] was injected into a 6 ft long 2% XE-60 column at 210 C with a carrier gas flow of 48 ml/min.

Isolation of the unknown steroid was achieved by the usually followed methods of steroid conjugate extraction [4] from 5·5 l of pooled urine specimen from the subject, hydrolysis with β-glucuronidase and preliminary separation on Whatman 3 mm papers in the solvent system benzene light petroleum (b.p. 100–120 C) methanol–water (250:250:300:200 by vol.) [4, 5]. The hydrolysis of the urine extract, although unnecessary in the current context, was carried out in the routine way prior to complete structural understanding of the substance. The substance was further purified by two preparative silica gel t.l.c. (1st system) benzene–ethyl acetate (1:1 v/v); 2nd system chloroform–methanol–water (74:26:2 by vol.). Finally, the substance was processed for acetylation and run on a system benzene–ethyl acetate (4:1 v/v) on silica gel t.l.c. The "acetate" product of the substance was clearly separated from other contaminating compounds and gave a single spot. After elution and hydrolysis with methanolic potassium hydroxide the substance gave a product which had the same R_f as the "acetate" derivative. The eluate gave a colourless gum (less than 0·5 mg) after vacuum evaporation. The substance has not been crystallized.

CHARACTERIZATION

The isolated material was identical with the authentic material in the following respects:

1. *Thin layer chromatography.* A distinct spot with phosphomolybdic acid reagent having an R_f of 0·15 on silica gel t.l.c. (benzene:ethyl acetate (3:2 v/v)) was given by the substance. The acetate, formate and trimethylsilyl ether derivatives showed the same R_f as the free substance indicating that it contained no reactive hydroxyl group.

2. *Gas chromatogram.* When gas chromatograms of a series of known reference steroids were examined for comparison, 1,4-androstadiene-3,17-dione showed similar retention time. The TMSE derivative and the free substance demonstrated the same retention time, confirming that it contained no reactive hydroxyl group.

3. The U.V. analysis of the substance showed an intense absorption at 242 nm, which can result from π π* transitions (K-band) of an α,β-unsaturated ketone.

4. The I.R. spectrum of the substance showed the following characteristics: the I.R. bands in the fingerprint region are barely resolved and are almost useless for the identification of the compound. However, two intense bands at 5·98 and 5·73 μm are characteristic for C=O stretching vibrations of α,β-unsaturated cyclohexanones respectively cyclopentanones or cyclohexanones.

5. The recording of the circular dichroism spectra on a Roussel–Jouan CD 185 Dichrograph showed four characteristic Cotton effects of alternating signs at 332 nm (−), 296 nm (+), 260 nm (−) and 231 nm (+). For disymmetrically disturbed α,β-unsaturated ketones a Cotton effect is generally found in the spectral range between 230 and 260 nm. Our substance in fact had two Cotton effects of relatively strong intensity at 260 nm (−) and 231 nm (+). The significant positive effect at 196 nm may be due to n–π* transitions of an isolated carbonyl group. These chiroptical properties indicated that the substance probably contained an α,β-unsaturated ketone, and a more or less isolated additional keto group at position A/B trans 3-, 11-, 12-, A/B cis 7- and 17.

6. Proton magnetic resonance spectra were recorded (Fig. 1) on a Bruker HFX-90-NMR spectrometer equipped with a Fourier transform unit. Generally for a good quality NMR spectrum about 30 mg of substance is required. Our isolated substance

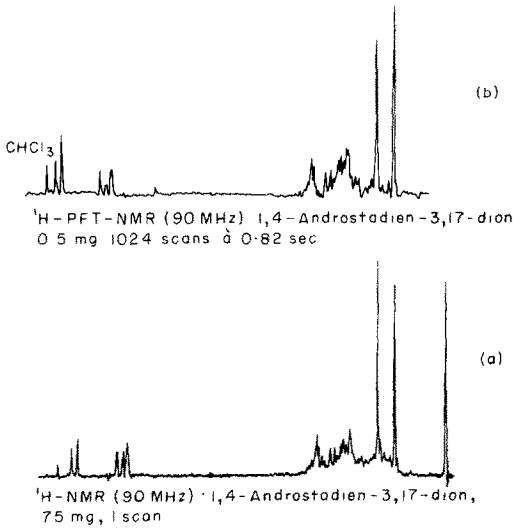

Fig. 1. 90 MHz ^{1}H-NMR spectra of 1,4-androstadiene-3,17-dione in CCl_4 solutions. (a) CW spectrum, single scan, amount of substance 75 mg in 0·4 ml CCl_4. (b) PFT spectrum, 1024 scans, amount of substance 0·5 mg in 0·4 ml CCl_4.

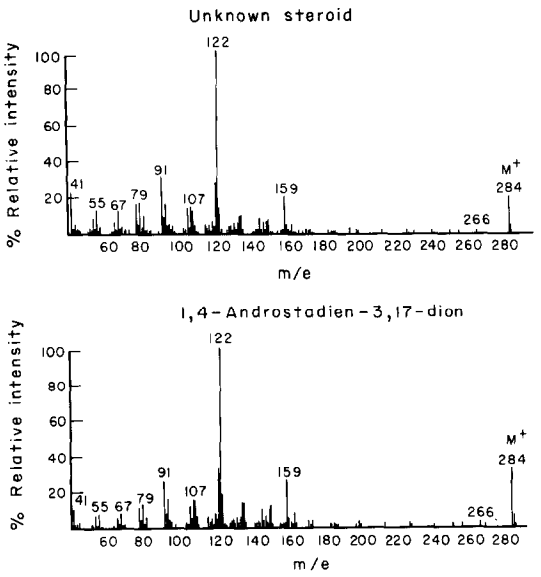

Fig. 2. Mass spectra of the isolated substance (a) and pure 1,4-androstadiene-3,17-dione (b). The spectra have been recorded on a GC-MS instrument (LKB 9000) under identical conditions.

was far too short of this quantity, and therefore, 124,000 interoferograms were accumulated in a Fabritek 1074 computer. Subsequent Fourier transformation in a PDP-8-1 computer revealed some useful signals which were found to be characteristic for methyl protons of the CH_3 groups in α,β-unsaturated 3-keto steroids.

7. The spectrum of the substance on a GC–MS instrument (LKB 9000) revealed that the free substance has an intense molecular peak at mass 284 and a characteristic peak at mass 122 (Fig. 2), which is the base peak. This latter fragment of m/e 122 has been frequently observed in 3-oxo-steroids. From several selected steroids with a molecular weight of 284 only 1,4-androstadiene-3,17-dione showed identical spectroscopic characteristics compared to the isolated compound.

DISCUSSIONS

The finding of 1,4-androstadiene-3,17-dione in the urine of the present subject was incidental to other studies and no systematic analysis of urine of this individual under functional tests was made.

Prior to the present isolation of this steroid, only one naturally occurring [Δ^1] steroid had been previously characterized [8]. This steroid 5α-androst-1-ene-3,17-dione, was reduced *in vivo* to both androsterone and epiandrosterone [9]. Ofner *et al.*[10] demonstrated the formation of 17α-OH-5α-androst-1-en-3-one when testosterone was incubated *in vitro* with human prostatic slices. Gawienowski *et al.*[11] observed the formation of 1,4-androstadiene-3,17-dione when progesterone was incubated *in vitro* with polycystic bovine ovary. Unger and Dorfman[9] demonstrated the reduction of Δ^1 double bond in C_{19} steroids *in vivo* in humans. The urinary metabolites of synthetic 1,4-androstadiene-3,17-dione given orally to human included a number of unsaturated (Δ^1 presumably) steroids not further identified. In connection with the biosynthetic pathways of 17β-oestradiol, the problem of the C-1 hydrogen atom involves the question of when in the reaction sequence it is eliminated, and how it is aromatized. Gual *et al.*[12] found that 25% of the synthetic 1,4-androstadiene-3,17-dione is converted to oestrogen. Our subject was under prolonged therapy of phenobarbital and diphenylhydantoin. It is known that treatment of rats with phenobarbital for as little as 4 days increases several fold the 7α, 6β, 6α and 2β hydroxylase activities for testosterone in the liver microsome fraction [13]. It is not known, however, whether this drug also activates the 1-hydroxylases. Diphenylhydantoin on the other hand decreases the magnitude of corticosteroid response to metopirone, perhaps by blocking pituitary release of ACTH in humans [14]. The extremely low values of the 17-OH-corticosteroids seen in our patient may be due to the effects of diphenylhydantoin. But the question of whether any of these drugs given over a considerable length of time may influence the 1,2-dehydrogenation of testosterone in man remains to be answered. The biological role of the substance is also not clear.

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