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IDENTIFICATION OF 3α-HYDROXY-5β-PREGNANE-11, 20-DIONE (11-OXOPREGNANOLONE) IN THE URINE OF HIRSUTE WOMEN USING MASS SPECTROMETRY AND GLC

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SUMMARY

3α-Hydroxy-5β-pregnane-11,20-dione has been identified in the urine of four hirsute women collected following the administration of corticotropin (ACTH), and in urine collected under basal conditions from one of these women. Lesser amounts were detected in the urine of two control subjects following ACTH administration. The steroid was identified principally on the basis of gas–liquid chromatography and combined gas chromatography–mass spectrometry. It is suggested, for one patient in particular, that the amount of this steroid present in the urine is further evidence for a partial defect of the adrenal 21-hydroxylase enzyme system.

INTRODUCTION

During the investigation by Fleetwood *et al.* [1] of a group of 28 hirsute women involving the measurement of certain urinary steroids, an unidentified substance was observed on the gas–liquid chromatograms of extracts of urine from some women. Extracts of urine for gas–liquid chromatography were prepared as in the procedure described by Ismail and Harkness [2] for assay of testosterone in urine. This procedure involved acid hydrolysis of steroid conjugates, extraction of the free steroids with diethyl ether, fractionation using the Girard reaction, alumina column chromatography, and paper chromatography.

The unidentified substance was present in urine specimens collected under basal conditions from one hirsute woman, and was observed in urine specimens collected on the second of two successive days of corticotropin (ACTH) administration from four patients. Smaller amounts were also detectable following ACTH stimulation of the adrenal glands in urine specimens from two of the ten control subjects. In the case of the hirsute patient in whose basal urine specimen the unidentified substance was observed there appeared to be a 6-fold increase in its excretion resulting from ACTH stimulation.

The circumstantial evidence outlined above suggested that the unidentified substance might well be a steroid of adrenal origin. This communication describes its characterization as 3α-hydroxy-5β-pregnane-11,20-dione, using principally gas–liquid chromatography (g.l.c.) and combined gas chromatography–mass spectrometry (g.c.–m.s.).

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EXPERIMENTAL

Gas–liquid chromatography was performed using a Varian 2100 instrument fitted with flame ionization detector. 150 cm columns of 1% QF-1 on Gas Chrom Q and 1% SE-30 on Gas Chrom Q were employed, at column temperatures of 213 and 210°C, respectively. Nitrogen was used as carrier gas (flow rate 25 ml/min) with both columns.

Mass spectra were recorded at an electron energy of 70 eV using an LKB 9000 gas chromatograph–mass spectrometer and a Du Pont 490F mass spectrometer linked to a Varian 2700 gas chromatograph via a single-stage glass jet separator.

RESULTS

Chromatographic data

The unidentified substance had the same mobility as testosterone in the paper chromatographic (p.c.) system toluene/petroleum spirit 60°–80°/methanol/water (33:66:80:20).

Using g.l.c., evidence was obtained that the unknown formed a trimethylsilyl ether derivative, and the retention increment suggested the presence of one hydroxyl group in the molecule.

Mass spectrometry

The mass spectrum of the trimethylsilyl ether derivative of the unidentified peak was recorded by g.c.–m.s. at $I^{235}_{OV\text{-}1}$ 2760 as shown in Fig. 1(a). In addition to the base peak at *m/e* 43 the main features of this mass spectrum were the molecular ion (*m/e* 404) and ions at *m/e* 260, 299 and 314. A metastable ion peak was recorded at *m/e* 284.7 (314–299). The

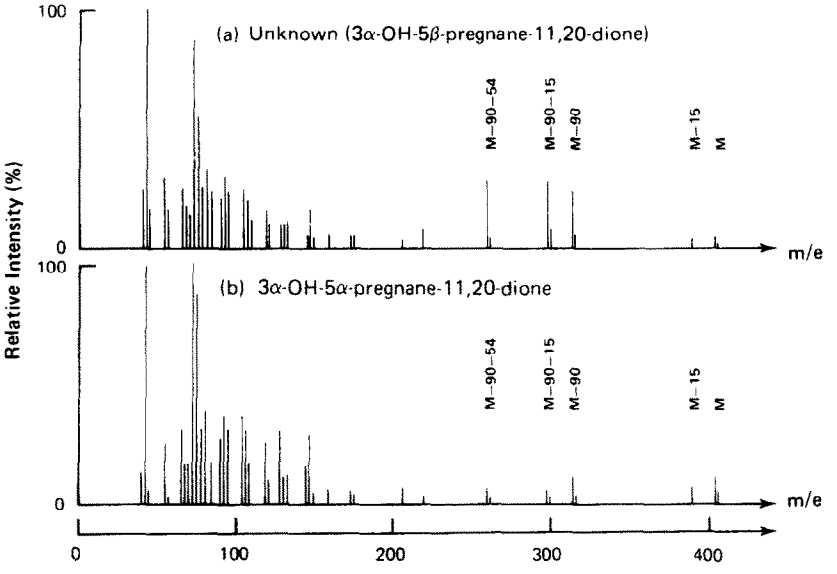

Fig. 1. Mass spectra (70 eV) of the trimethylsilyl ethers of (a) the unknown, and (b) 3α-hydroxy-5α-pregnane-11,20-dione.

molecular ion indicated a molecular formula of $C_{21}H_{32}O_3$ for the parent steroid (mol. wt 332). The base peak at *m/e* 43 was consistent with a 17-acetyl side-chain. This was confirmed by formation of an *O*-methyloxime trimethylsilyl ether, the mass spectrum of which showed a molecular ion at *m/e* 433 and a base peak at *m/e* 100, typical of 20-ketopregnane *O*-methyloximes [3]. The formation of only a mono-*O*-methyloxime mono-trimethylsilyl ether suggested that the third oxygen atom was present as an unreactive ketone. The most common site for such a group is C-11, and since 11-hydroxylase and 11-hydroxy oxido-reductase activities occur in the human adrenal cortex, it seemed probable that the the steroid was a 3-hydroxypregnane-11,20-dione. Further major peaks in the mass spectrum of the trimethylsilyl ether were consistent with this, as illustrated in Fig. 2. In particular, the ion at *m/e* 85, attributable to cleavage of ring D as shown, indicated that the ketonic oxygen atom was not at C-15 or C-16. Loss of the elements of trimethylsilanol, and breakdown of ring A by "retro-Diels-Alder" fragmentation (cf. Loudon [4]), are characteristic of 3-hydroxypregnane trimethylsilyl ethers. The high abundance of the ion resulting from the "retro-Diels-Alder" cleavage of ring A was suggestive of the 5β -configuration, as indicated (*inter al.*) by the data of Ende and Spiteller [5] for the free 3-hydroxy-pregnane-11,20-diones.

One of the four possible isomers, 3α-hydroxy-5α-pregnane-11,20-dione, was initially available for comparison. The retention time of its trimethylsilyl ether ($I^{235}_{OV\text{-}1}$ 2750) was distinctly lower than that of the unknown. The mass spectrum, shown in Fig. 1(b), exhibited a close similarity to that of the unknown in respect of the peaks at *m/e* 260, 299, 314 and 404, and also in the positions of peaks resulting from ions of lower *m/e* ratios. However, there were significant differences in the relative intensities of the major peaks. From a comparison of the g.l.c. data and mass spectra it therefore appeared that the parent steroid had a structure very similar to 3α-hydroxy-5α-pregnane-11,20-dione, and was probably a stereoisomer thereof.

Gas–liquid chromatography

In order to confirm the structure of the unknown as a 3-hydroxypregnane-11,20-dione, and to identify the configurations at C-3 and C-5, g.l.c. using two

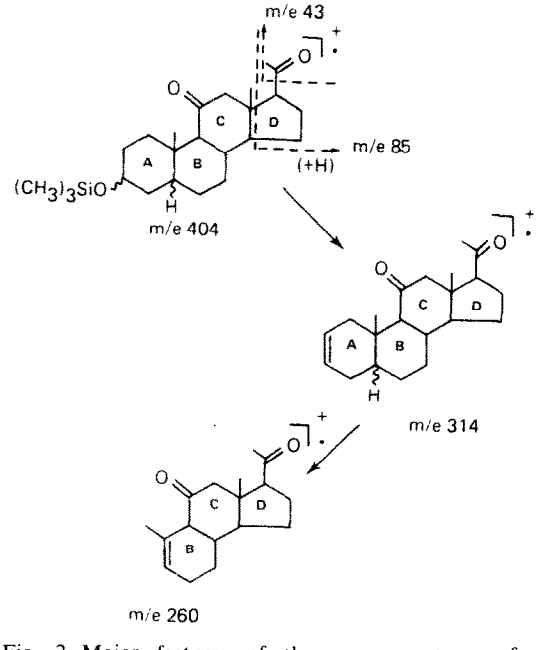

Fig. 2. Major features of the mass spectrum of a 3-hydroxypregnane-11,20-dione trimethylsilyl ether.

Table 1. Gas–liquid chromatography relative retention times of unknown steroid and isomers of 3-hydroxypregnane-11,20-dione

Steroid chromatographed	Retention time relative to testosterone	
	Column of 1% QF-1 Temp. = 213°	Column of 1% SE-30 Temp. = 210°
3α-hydroxy-5α-pregnane-11,20-dione	1.53	1.65
3β-hydroxy-5α-pregnane-11,20-dione	1.84	1.67
3α-hydroxy-5β-pregnane-11,20-dione	1.55	1.43
3β-hydroxy-5β-pregnane-11,20-dione	1.48	1.47
Unknown steroid	1.55	1.43

different liquid phases was employed. The respective retention times of each of the four possible isomers (relative to testosterone) on columns of 1% QF-1 and 1% SE-30 are given in Table 1, and compared to those of the unidentified substance. These data showed that 3α-hydroxy-5β-pregnane-11,20-dione had identical relative retention times to those of the unknown in both g.l.c. systems.

Confirmatory evidence (g.l.c. and g.c.–m.s.)

The trimethylsilyl ethers of 3α-hydroxy-5β-pregnane-11,20-dione and the unknown were compared using g.l.c. and were found to have the same relative retention times ($I_{OV\cdot1}^{235} = 2760$). The oxidation product of the unknown was found to have an identical g.l.c. retention time to the oxidation product of 3α-hydroxy-5β-pregnane-11,20-dione, and to differ from that of the oxidation product of the 5α-pregnane isomers. The unknown was found to have the same p.c. mobility as 3α-hydroxy-5β-pregnane-11,20-dione in the system toluene/petroleum spirit 60°–80°/methanol/water (33:66:80:20).

Finally, a mass spectrum of the trimethylsilyl ether of 3α-hydroxy-5β-pregnane-11,20-dione was obtained by g.c.–m.s. and compared to that of the unknown. The spectra, recorded at the same retention times, were virtually identical in all respects. It was concluded that the originally unidentified peak consisted of 3α-hydroxy-5β-pregnane-11,20-dione.

Quantitation

It was estimated that the excretion of 3α-hydroxy-5β-pregnane-11,20-dione in the basal urine specimen of one hirsute woman (patient 9, [1]) was 0.3 mg/24 h, and for this patient the "post-ACTH excretion" was estimated to be 1.9 mg/24 h. The compound was not present in basal urine specimens from any of the other 27 patients, but for three of these women the "post-ACTH excretion" was 0.17, 0.22 and 0.13 mg/24 h, respectively.

11-oxopregnanolone was not present in basal urine specimens from any of the ten control subjects, but for two of these subjects small amounts (0.04 and 0.06 mg/24 h) were present in urine specimens collected following ACTH administration.

DISCUSSION

3α-hydroxy-5β-pregnane-11,20-dione (11-oxopregnanolone) has previously been identified in human urine [6]. These investigators isolated the compound from the urine of a child suffering from congenital adrenal hyperplasia due to a defect in the adrenal 21-hydroxylase enzyme system. Although the predominant urinary excretion products in patients with an adrenal 21-hydroxylase defect are pregnanetriol and 17α-hydroxypregnanolone, it is recognized that the excretion of the corresponding 17-deoxy steroids, pregnanediol and pregnanolone, is also elevated to a lesser extent in this condition [7–9]. Several authors have reported excessive amounts of the 11-oxo derivative of pregnanetriol, 11-oxopregnanetriol, in the urine of patients with an adrenal 21-hydroxylase deficiency [10, 7, 8], and the corresponding 17-deoxy compounds, 11-oxopregnanediol and 11-oxopregnanolone, have also been shown to occur in urine in this condition [6, 7]. 11-oxopregnanolone is therefore considered to be one of a group of steroids not normally detected in urine, but present in the urine of patients with the 21-hydroxylase type of congenital adrenal hyperplasia [9].

In the present study 11-oxopregnanolone was detected in considerably larger amounts in the urine of patient 9 than in the urine of any other subject. Patient 9 had previously been investigated using the "ACTH-metyrapone" test [1], and from the urinary pregnanetriol response to this test was suspected to be a heterozygote for the adrenal 21-hydroxylase enzyme defect. In fact this patient gave by far the most exaggerated response to the ACTH-metyrapone test of any patient studied, and also had the highest basal urinary pregnanetriol excretion. In addition, patient 9 had the highest basal excretion of urinary testosterone and urinary 11-deoxy-17-oxosteroids. The 11-oxopregnanolone results of patient 9 were therefore considered to be in accord with the other evidence which indicated that this patient might have a moderately severe biochemical defect of the adrenal 21-hydroxylase enzyme system.

The estimated post-ACTH excretion of urinary 11-oxopregnanolone by the other patients in whose urine this compound was detected was also greater

than that of any control subject. Patients 1 and 4 had previously been investigated using the ACTH-metyrapone test, and in both cases the urinary pregnanetriol response to this test procedure had indicated that a partial deficiency in the adrenal 21-hydroxylase enzyme system might be operative in these two patients also.

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