Notes

CHROM. 5326

A new type of micro-analysis of steroid hormones in biological materials by gas chromatography with flame ionisation and electron capture detection

I. The urinary profile

For the gas phase analysis of a mixture of steroids in biological materials, steroid derivatives stable under the temperature conditions of gas chromatography (GC) are necessary. O-Methyloxime-trimethylsilyl derivatives (MO-TMSi) introduced by GARDINER AND HORNING[1] in 1966 allowed them to perform the so-called "steroid biological profile" with flame ionisation detection (FID). Electron capture detection (ECD) brings GC sensitivity to the nanogram and even the subnanogram level using heptafluorobutyrate derivatives, but these derivatives, depending on the type of steroid, are not always stable in GC. With 17-desoxycorticosteroid, and more extensively with 17-hydroxycorticosteroid esters, multiple peaks appear which probably involve side-chain cleavage[2], whilst with a ketosteroid, like dehydroepiandrosterone (DHEA), we found that the 17-keto group is partially enolised leading to two derivatives. If methyloxime formation is carried out before the perfluoroacylation, stable derivatives are obtained and enolisation is prevented. In 1969, HORNING AND MAUME[3] studied O-methyloxime-heptafluorobutyrate derivatives (MO-HFB) for aldosterone. The formation of these derivatives applied to other steroids and their metabolites allows good thermal stability and sensitivity at the nanogram level.

Besides being used for biological fluids containing a small amount of steroid, this method appears very interesting for steroid analysis on small tissue samples in which steroid metabolism exists. Because it has been shown that adrenal tumor cells grown in culture[4] and adrenal cell suspension[5] synthesize steroids[5,6], our method should be valid for a more specific and complete study of steroidogenesis in the course of cell differentiation or development. Following the work of HARARY AND FARLEY[7] showing that single normal heart cells can be kept and grown in Petri dishes, recent progress in maintaining normal non-embryonic cells from organs related to steroid metabolism, like the liver[8,9] and adrenal glands[10], prompted us to ascertain the validity of our analytical method. Therefore for comparison with other methods, this preliminary work has been done on human urine, the most investigated mixture of steroids.

Methods

For the standards, the formation of MO-HFB derivatives is described elsewhere[11]. O-Methyloxime (MO) derivatives were made by the usual method[12]. Excess reagent was eliminated by washing with a sodium chloride solution. Heptafluorobutyrylimidazole was used as the reagent for HFB derivative formation. These derivatives were then extracted by hexane at —18°. The final amount injected was 1 μl containing 1 μg for FID and 1 ng for ECD.

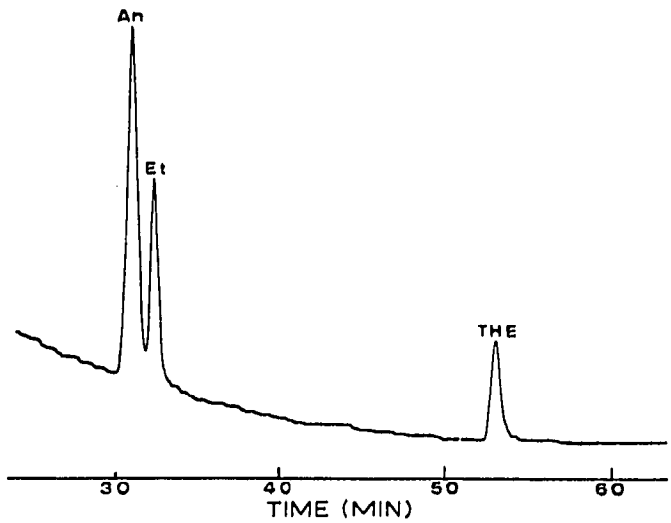

Fig. 1. Separation of a synthetic mixture of MO-HFB steroids on a 12-ft. 1% OV-1 column made according to HORNING *et al.*[16]. Gas chromatograph is a model 7400 Packard with FID. Compounds are androsterone (An), etiocholanolone (Et) and tetrahydrocortisone (THE). Temperature settings: programming at 1°/min from 170 to 230°, 240° for the injection, 260° for the oven detector.

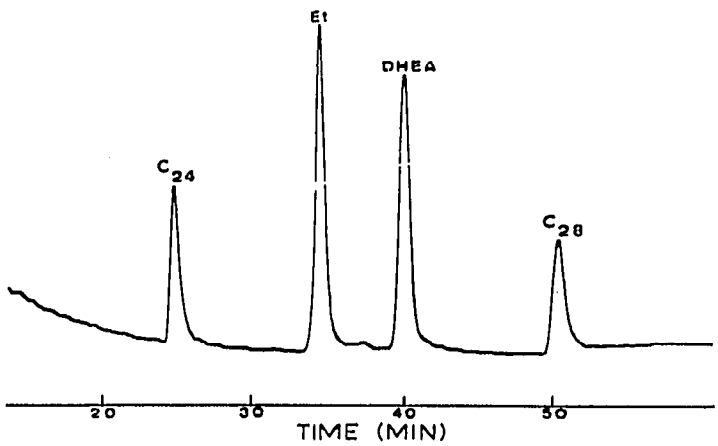

Fig. 2. Same as Fig. 1, but the separation was carried out on a 1% OV-17 column. Two alkanes (C_{24} = *n*-tetracosane, C_{28} = *n*-octacosane) were added as internal standards for methylene unit (MU) calculation.

TABLE I

METHYLENE UNIT VALUES (MU) OF HFB AND MO-HFB DERIVATIVES OF SOME HUMAN URINARY STEROIDS

Steroids	*OV-1*[a]	*OV-17*[a]
Androsterone (3α-hydroxy-5α-androstan-3-one)	24.07	25.47
Etiocholanolone (3α-hydroxy-5β-androstan-3-one)	24.25	25.55
Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one)	24.85	26.41
Pregnanediol[b] (3α,20α-dihydroxy-5β-pregnane)	25.69	25.15
Pregnanolone (3α-hydroxy-5β-pregnan-20-one)	26.04	27.36
Tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione)	27.52	28.08

[a] 12-ft. column; 1% liquid phase; temperature, 1°/min from 170°.
[b] HFB ester, other steroids are as MO-HFB derivatives.

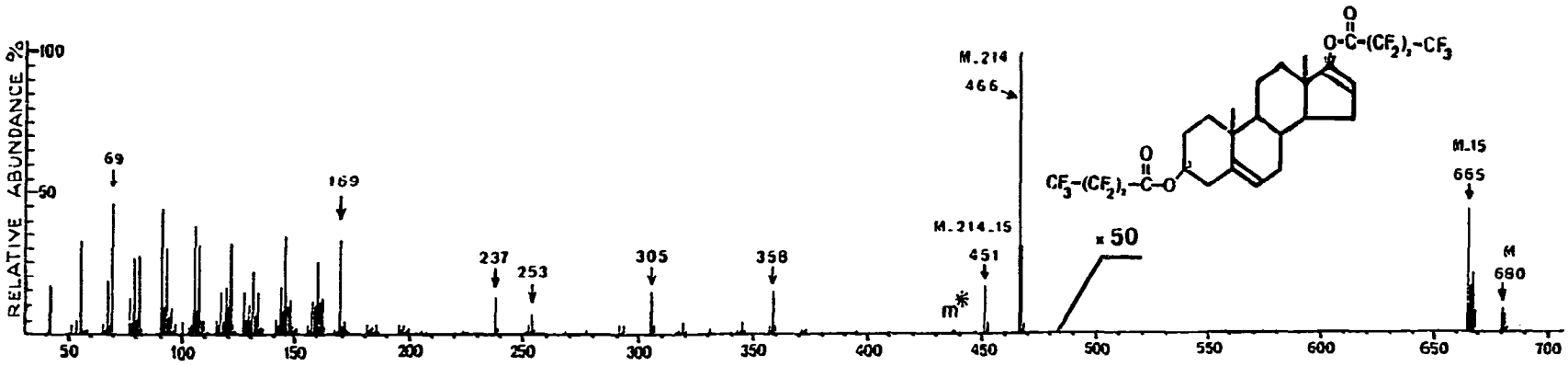

Fig. 3. Mass spectrum of 3β,17-(enol)-diHFB-dehydroepiandrosterone. Fragments providing structural information are m/e = 680 (M), 665 (M-15), 466 (M-214) corresponding to the loss of heptafluorobutyric acid from the molecular ion, 451 (M-214-15), 253 (M-214-213), 252 (M-2 × 214), 237 (M-2 × 214-15). Peaks at 69, 119 and 169 a.m.u. correspond to CF_3^+, $C_2F_5^+$, $C_3F_7^+$, respectively. Metastable ion found at m/e = 436.7 corresponds to the transition 466 $\xrightarrow{-15}$ 451 (calculated m/e = 436.48). This spectrum was obtained with an LKB 9000 gas chromatograph–mass spectrometer; ionising energy: 70 eV; ionisation source temperature 200°.

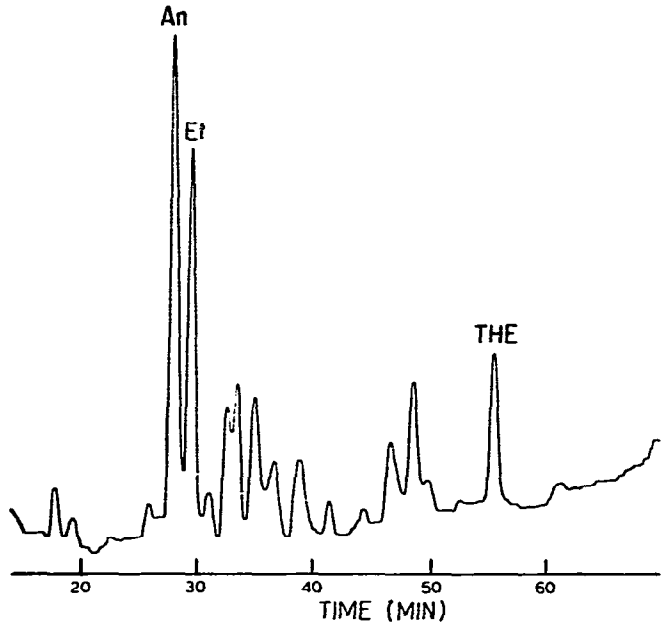

Fig. 4. Same conditions as Fig. 1. Separation of adult male urinary steroids.

25-ml urinary samples were each hydrolysed enzymatically, and the free steroids were transformed to MO-HFB derivatives by the previous method. Estrogens were separated by partition between aqueous ethanol (2 %) and benzene–hexane (1 : 1). The GC apparatus equipped with FID and ECD are described in the legend to Fig. 1.

Results and discussion

Figs. 1 and 2 show that MO-HFB derivatives are thermostable and each give a single peak. The side chain of C_{21} steroid-like tetrahydrocortisone is protected from cleavage by MO-HFB formation. If no MO formation occurs before perfluoroacylation, we have found that, in the case of 17-ketosteroid, HFB anhydride induces a partial enolisation of the keto group under the usual reaction conditions. For example, DHEA heated 1 h at 60° with HFB anhydride in acetonitrile (3 : 10) gives, besides the 3β-HFB derivative, a minor peak which has been identified by mass spectrometry as the 3β,17-diHFB (enol) derivative (Fig. 3). The second enol peak increases if the reaction time is longer (65 % of HFB enol after 15 h) (ref. 13). Although it may be satisfactory to obtain 100 % enol formation by reacting for 36 h at 60° instead of avoiding it, it is preferable, especially if acylation is carried out over a shorter time, to perform the MO reaction first.

The methylene unit values[14] of MO-HFB derivatives shown in Table I are smaller than those given by corresponding MO-TMSi[15].

Therefore faster elution is another interesting feature of these MO-HFB derivatives. Moreover the separation between isomeric steroids such as MO-HFB or HFB compounds is generally improved in comparison with other types of derivatives Examples are given for the separation between MO-HFB androsterone and MO-HFB etiocholanolone (Figs. 1 and 4) and more strikingly between isomeric HFB estriols[11] (Fig. 7).

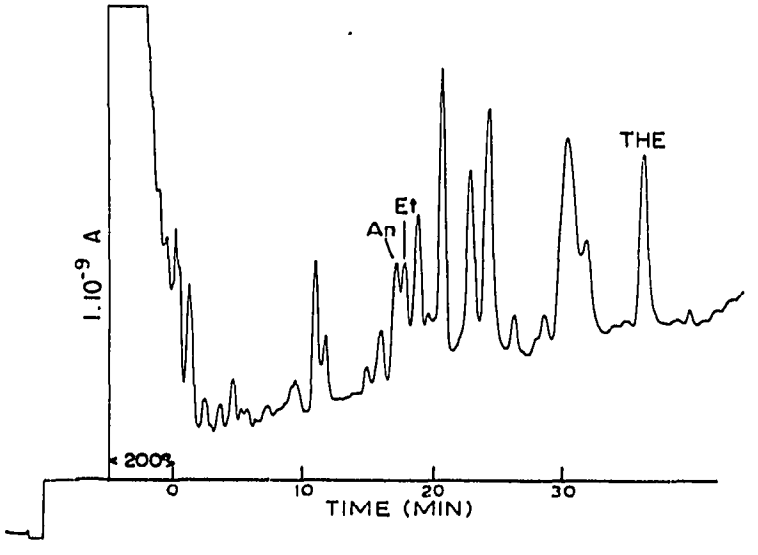

Fig. 5. Same urinary sample as in Fig. 4, but diluted to 1/1000. Column was a 6-ft. 1 % OV-1 under isothermal conditions at 200° for 5 min and then temperature programming at 1 °/min. Detection was done by ^{63}Ni-ECD with 2 V DC, the detector oven being set at 255°. Comparison with Fig. 4 shows a smaller relative response for "An" and "Et", bearing one HFB, than for THE bearing two HFB.

Fig. 4 shows a characteristic separation of male adult urinary steroids as MO-HFB derivatives with the use of FID. When the gas chromatograph is operated with ECD, relative heights of peaks are modified because the response of this detector is dependent on the number of fluorine atoms and consequently on the number of OH groups which have been esterified in the molecule (Fig. 5).

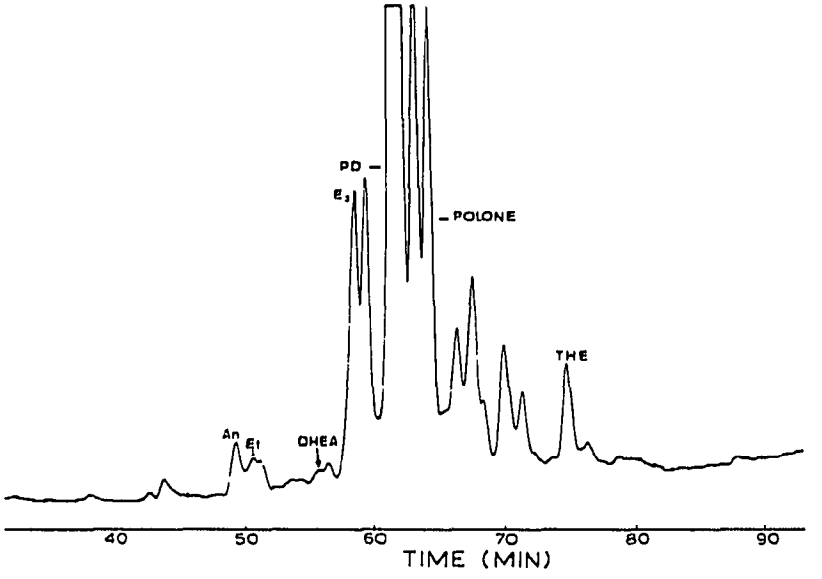

Fig. 6. Separation of MO-HFB steroids of a urinary sample from pregnant woman. Temperature programmed from 150° at 1°/min; other conditions as in Fig. 1. Other compounds identified are estriol (E3), pregnanediol (Pd), pregnanolone (Polone).

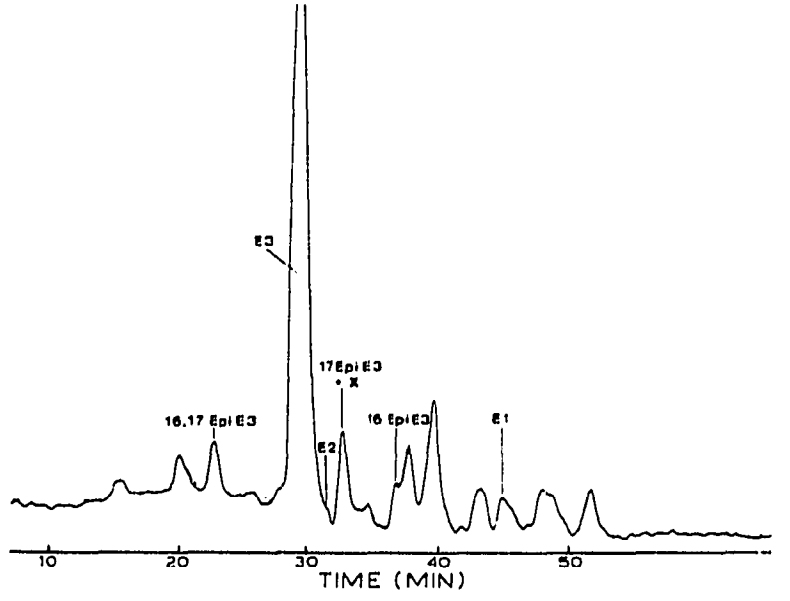

Fig. 7. Separation of HFB and MO-HFB steroids of a urinary sample from a pregnant woman in the last month of pregnancy. 6-ft. 1% OV-17 column; temperature programmed from 170° at 1°/min; ECD. See conditions in Fig. 5 for detection by electron capture. The amount injected was equivalent to 3 μl of the original urine sample. Compounds identified are E1 = estrone; E2 = 17β-estradiol; E3 = 3,16α,17β-estriol; 17epiE3 = 3,16α,17α-estriol; 16,17-epiE3 = 3,16β,17α-estriol; 16-epiE3 = 3,16β,17β-estriol.

Total urinary steroids at the end of the pregnancy of a woman are shown in Fig. 6 while the analysis of the estrogenic fraction of the same sample is shown in Fig. 7, ECD being used. Thanks to this method, six of the many possible estrogens have been identified. This technique improves the separation and the sensitivity of detection of minute amounts of isomeric estriols in a simple analysis of a total estrogenic fraction avoiding preliminary subfractionation. The complete structure of these derivatives and the homogeneity of each peak in biological profile are undertaken with the use of combined gas chromatography–mass spectrometry. The application of this method to the study of small amounts of steroids in plasma, for instance, or in a small amount of tissue sample, as in normal single cell adrenal[10] or liver[9] culture, is under development.

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