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BASE-CATALYZED SILYLATION. A QUANTITATIVE PROCEDURE FOR THE GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF NEUTRAL STEROIDS

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SUMMARY

TMS derivatives of the most important urinary neutral steroids were obtained by the utilization of a potassium acetate-catalyzed reaction. Samples were analyzed using a gas chromatograph-mass spectrometer equipped with a glass capillary column.

In addition to silylation of hydroxyl groups, this procedure promoted enolization of 20-ketosteroids in some instances and the corresponding enol-TMS were obtained.

Analysis of a variety of urinary neutral steroids in this manner revealed that, in nearly all cases, a unique derivative was formed which could be detected quantitatively by gas chromatography-mass spectrometry.

INTRODUCTION

MANY methods to convert steroids to appropriate derivatives for gas-chromatographic analysis have been described[1]. The most suitable and widely used derivatives are the trimethylsilyl ethers (TMS) of hydroxyl functions and the methyloximes of keto groups[2, 3]. A number of powerful silylating agents and special procedures have been proposed for the functionalization of hindered hydroxyl and ketonic groups[1]. However, most of these techniques are inconvenient for routine clinical assay, since they usually require more than one reagent, long reaction times and high temperatures. Such conditions may lead to alterations in the steroids present in biological tissues and fluids.

Chambaz *et al.*[4] have demonstrated that the dihydroxyacetone side-chain of some corticosteroids may be completely converted to a stable derivative, TMS-enol-TMS, by using N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) in the presence of potassium acetate as nucleophilic catalyst. On the basis of this finding, we extended this base-catalyzed reaction to the more common steroids present in human urine, not only to determine the nature of the products formed, but also to explore the possible utility of this approach for clinical analysis.

Individual urinary steroids and their mixtures were treated as above and analyzed by a gas chromatograph–mass spectrometer equipped with a glass capillary column to obtain a better resolution of gas chromatographic peaks. All of the important neutral steroids gave rise to a single derivative, and thus demonstrated that this procedure is a potentially useful and convenient method for clinical analysis of urinary steroids.

Mass spectra of TMS derivatives not previously reported are also discussed.

MATERIALS AND METHODS

Chemicals

Reference steroids were purchased from Vister, Casatenovo Brianza, Italy, Mann Research Laboratories, New York, N.Y., U.S.A., and from Ikapharm, Ramat-Gan, Israel. N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), specially purified grade, was purchased from Pierce Chemical Co., Rockford, Ill., U.S.A. 1,1,1,3,3,3,-Hexamethyl-disilazane (HMDS) was purchased from Schuchardt, München, Germany. Potassium acetate was purchased from B.D.H. Italia, Milan, Italy.

Silylation reaction

The silylation reaction was carried out in tightly capped small test tubes by dissolving the steroids in BSTFA (100 μl BSTFA/30 μg of steroids). In some cases, carbon disulphide was used to dissolve highly crystalline reference steroids. This was subsequently removed after addition of BSTFA by evaporation under a slow stream of nitrogen. Potassium acetate (0·5–1 mg/30 μg of steroids) was added and the tubes heated in an aluminium block at 60°C for 40 min. The BSTFA was evaporated under nitrogen, the residue dissolved in HMDS, and the solution kept in a desiccator at room temperature until analysis.

Gas chromatography–mass spectrometry

An LKB Model 9000 gas chromatograph–mass spectrometer (LKB Produkter AB., Stockholm) was used. This instrument was modified in this laboratory in order to use a glass capillary column; an additional helium stream, pre-heated at the temperature of the column oven was added just before the end of the column to have proper flow conditions in the separator[5].

Samples were injected according to the method of Ros[6] and modified for adaptation to the LKB 9000 instrument.

The column, purchased from Dr. Grob, Zürich, was a 50 m × 0·3 mm open tubular glass column. The stationary phase was OV-101 and the carrier gas was helium (2 ml/min through the column, 40 ml/min through the separator). The temperature programme was: 20 min at 190°C, 2°C/min, up to 250°C. The flash heater, separator and ion source temperatures were 270, 280 and 290°C, respectively.
Electron energy: 70 eV
Trap current: 70 μA
Accelerating voltage: 3·5 kV

The gas chromatographic curve was recorded monitoring the total ion current with 20 eV electron energy.

RESULTS AND DISCUSSION

A systematic analysis of the derivatives obtained from neutral urinary steroids under conditions described in methods has been carried out using gas chromatography-mass spectrometry. The use of a glass capillary column[5] allowed the complete resolution of reaction products[6]. The quantitative aspect of the reaction was estimated by reference to two stable external standards, cholesteryl butyrate and cholestane. Reproducible response coefficients (close to one) were regularly obtained for all the steroid tested. In all but two cases, a single chromatographic peak was produced by the steroid standards. 11-O-An and 11-O-Et, on the other hand, generated two peaks of almost equal intensity. Moreover, the two

derivatives produced by 11-O-Et showed different retention times from that of all the other urinary steroid derivatives. The retention time of the first peak derived from 11-O-An was also unique, whereas the second peak showed the same retention time as the peak obtained from Pn. This latter finding would not appear to invalidate the method, however, because 11-O-An is present only as a trace in urinary neutral steroids.

Mass spectrometry established the number and, in most cases, the position of the derivatized hydroxy and keto groups; the results of such analysis are shown in Table 1.

The hydroxyl groups in position 3 (α or β) and 20α reacted completely, as

Table 1.

Abbreviation	Trivial name	Systematic name	Silylated function	Molecular weight	Relative retention time†
An	Androsterone	3α-hydroxy-5α-androstan-17-one	3α	362	0·334
Et	Aetiocholanolone	3α-hydroxy-5β-androstan-17-one	3α	362	0·355
DHA	Dehydroepiandrosterone	3β-hydroxy-5-androsten-17-one	3β	360	0·390
epi-An	Epiandrosterone	3β-hydroxy-5α-androstan-17-one	3β	362	0·400
11-O-An	11-Oxo-Androsterone	3α-hydroxy-5α-androstane-11,17-dione	3α 3α, 17	376 448	0·402 0·497‡
11-O-Et	11-Oxo-aetiocholanolone	3α-hydroxy-5β-androstane-11,17-dione	3α 3α, 17	376 448	0·415 0·502‡
11-OH-An	11-Hydroxyandrosterone	3α,11β-dihydroxy-5α androstan-17-one	3α	378	0·472
11-OH-Et	11-Hydroxyaetiocholanolone	3α,11β-dihydroxy-5β-androstan-17-one	3α	378	0·487
Pn	Pregnanolone	3α-hydroxy-5β-pregnan-20-one	3α	390	0·497
a-Pd	allo-Pregnanediol	5α-pregnane-3α,20α-diol	3α, 20α	464	0·590
Pd	Pregnanediol	5β-pregnane-3α,20α-diol	3α, 20α	464	0·610
5-Pd	5-Pregnenediol	5-pregnene-3β,20α-diol	3β, 20α	462	0·640
Pt	Pregnanetriol	5β-pregnane-3α,17α,20α-triol	3α, 20α	480	0·713
5-Pt	5-Pregnenetriol	5-pregnene-3β,17α,20α-triol	3β, 20α	478	0·745
THS		3α,17α,21-trihydroxy-5β-pregnan-20-one	3α, 17α 20, 21	638	0·765
Pt-olone	Pregnanetriolone	3α,17α,20α-trihydroxy-5β-pregnan-11-one	3α, 20α	494	0·780
THE	Tetrahydrocortisone	3α,17α,21-trihydroxy-5β-pregnane-11,20-dione	3α, 17α 20, 21	652	0·845
THF	Tetrahydrocortisol	3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one	3α, 17α 20, 21	654	0·857
THA	Tetrahydrodehydrocorticosterone	3α,21-dihydroxy-5β-pregnane-11,20-dione	3α, 20, 21	564	0·863
THB	Tetrahydrocorticosterone	3α,11β,21-trihydroxy-5β-pregnan-20-one	3α, 20 21	566	0·885
a-THB	allo-Tetrahydrocorticosterone	3α,11β,21-trihydroxy-5α-pregnan-20-one	3α, 21,20	566	0·890

Table 1 (cont.)

Abbreviation	Trivial name	Systematic name	Silylated function	Molecular weight	Relative retention time†
	Cortolone	3α,17α,20α,21-tetra-hydroxy-5β-pregnan-11-one	3α*	582	0·917
	β-Cortolone	3α,17α,20β,21-tetra-hydroxy-5β-pregnan-11-one	3α*	582	0·931
	Cortol	5β-pregnane-3α,11β,17α, 20α,21-pentol	3α*	584	0·951
	β-Cortol	5β-pregnane-3α,11β-17α, 20β,21-pentol	3α*	584	0·961
	Cortisone	17α,21-dihydroxy-4-pregnene-3,11,20-trione	17α, 20, 21	576	0·949
	Corticosterone	11β,21-dihydroxy-4-pregnene-20-dione	20,21	490	0·956
	Cortisol	11β, 17α, 21-trihydroxy-pregnene-3,20-dione	17α, 20, 21	578	0·988
CB	Colesteryl butyrate				1·000

*Two out of three hydroxy groups in the side-chain are silylated.

†Gas chromatographic conditions are described under "Materials and Methods". R_f of cholesteryl butyrate was 78 min.

‡See text.

demonstrated by the molecular ions in the mass spectra of derivatized Pd, a-Pd ($M^+ = 464$) (Fig. 1), and 5-Pd ($M^+ = 462$)[7] which showed the presence of two TMS groups. Moreover, the base peak at m/e 117 corresponded to the side chain containing a TMS derivative in position 20 (α or β)[7].

The peak at m/e 117 was also present in the mass spectra of the derivatives of Pt, 5-Pt, and Pt-olone (Fig. 2), which showed M^+ values ($M^+ = 480$, 478, 494

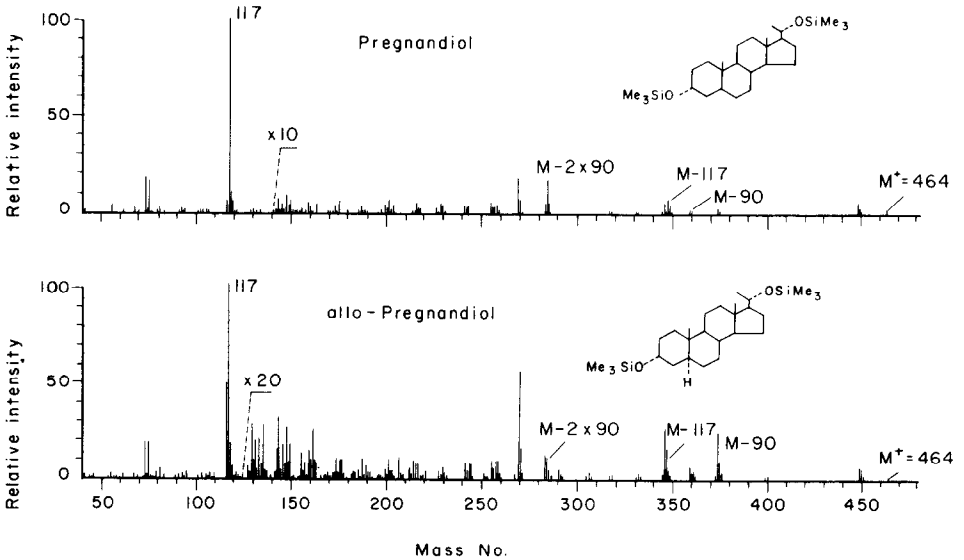

Fig. 1. Mass spectra of the Pd and α-Pd derivatives.

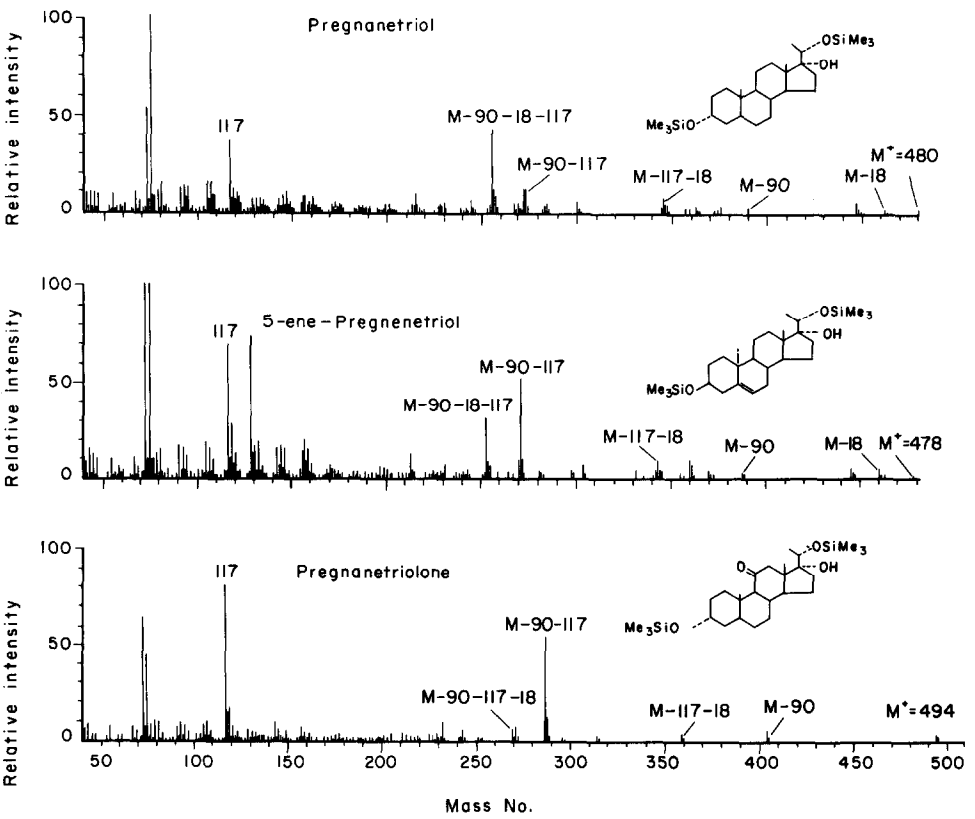

Fig. 2. Mass spectra of the Pt, 5-Pt and Pt-olone derivatives.

respectively) corresponding to the presence of two TMS groups (in position 3 (α or β) and 20α); this indicated that the 17α-tert-hydroxyl group remained free. Enolization and subsequent silylation of 17-keto androstanes was not observed since An ($M^+ = 362$), epi-An ($M^+ = 362$), Et ($M^+ = 362$) and DHA ($M^+ = 360$) showed molecular ions corresponding to a mono-TMS derivative. These are the same products obtained under normal conditions for silylation[7].

The 11β-hydroxyl group was also unaffected under the described condition. For example, the derivatives of 11-OH-An and 11-OH-Et displayed the same M^+ value ($M^+ = 378$) corresponding to the mono-TMS derivative. Moreover, their mass spectra were similar to those for silylated An and Et[7], except for the loss of 18 mass units from the most prominent peaks. Similarly, the 11-keto group remained undisturbed, as illustrated by Pt-olone (Fig. 2).

In contrast to the above results, 11-O-An and 11-O-Et (Fig. 3) displayed quite a different behaviour towards silylation. In both cases, partial enolization of the 17-keto group occurred giving rise to a mixture of the mono and bis-TMS ethers.

The mass spectrum of the mono-TMS derivative of 11-O-An showed two peaks, which is consistent with the presence of a TMS group in position 3; the first one (m/e = 232) corresponded to a Retro Diels–Alder reaction on the A ring after loss of trimethylsilanol, and the second one (m/e = 320) corresponded to loss of the D ring from the molecular ion. The fragment at m/e = 232 was also present in the mass spectrum of the mono-TMS derivative of 11-O-Et (Fig. 3); however, its relative intensity was much higher. The mass spectra of the bis-

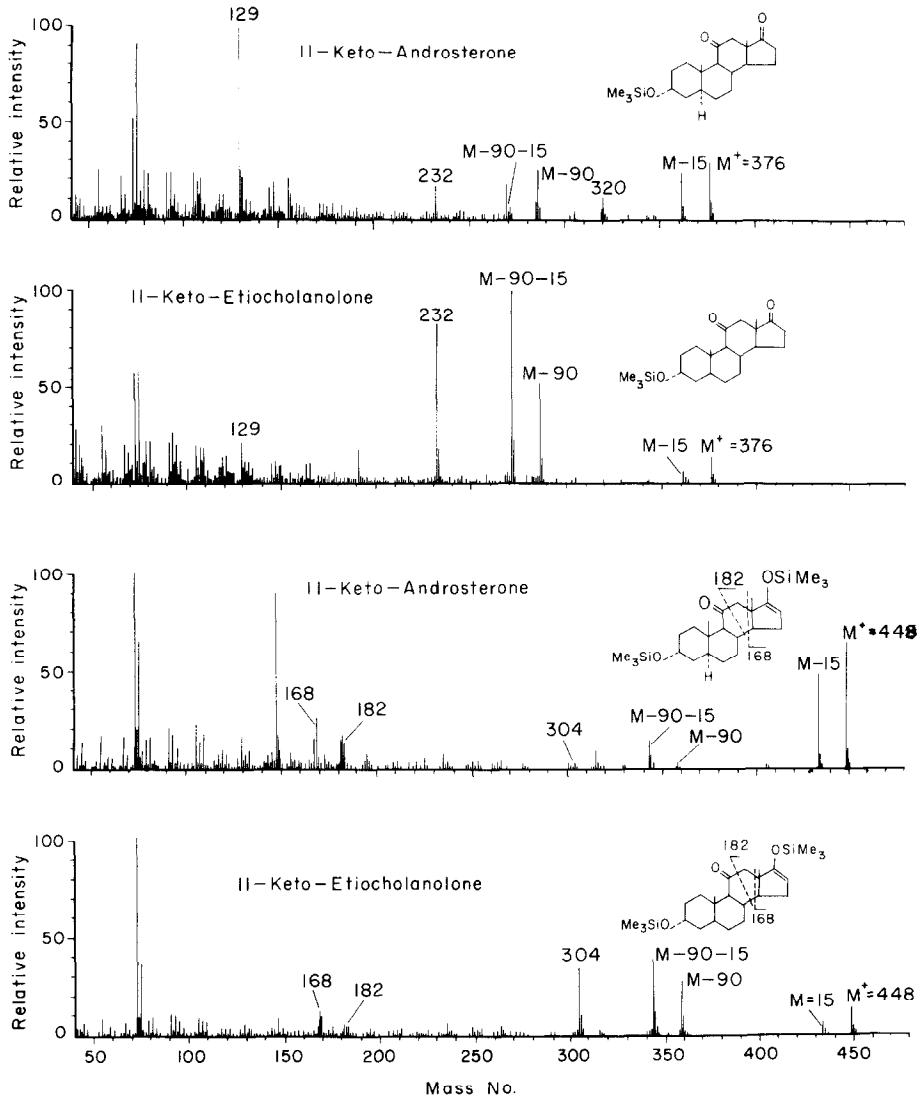

Fig. 3. Mass spectra of the mono and bis derivatives of 11-O-An and 11-O-Et.

TMS derivatives of both 11-O-An and 11-O-Et (Fig. 3) showed peaks at m/e 168, 169 and 182 indicating that an enol-TMS group was present in position 17.

The above described Retro Diels–Alder reaction generated in this case a fragment at m/e 304, the identity of which was confirmed by the presence of a metastable ion (M* = 258·1) in the spectrum of the bis-TMS derivative of 11-O-Et.

In contrast to other silylation procedures for steroids, the base-catalyzed reaction (described here) promoted the reactivity of the 20-ketone function of steroids in many instances depending on the presence of other functions. The C-20 ketone was completely inert when it was the only function present in the pregnane lateral chain. Thus Pn showed a molecular ion ($M^+ = 390$) corresponding to the mono-TMS derivative (Fig. 4). The fragments at m/e 257, 230 and 215

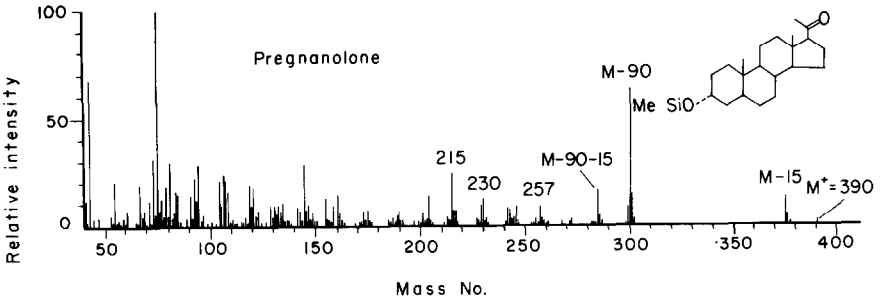

Fig. 4. Mass spectrum of the Pn derivative.

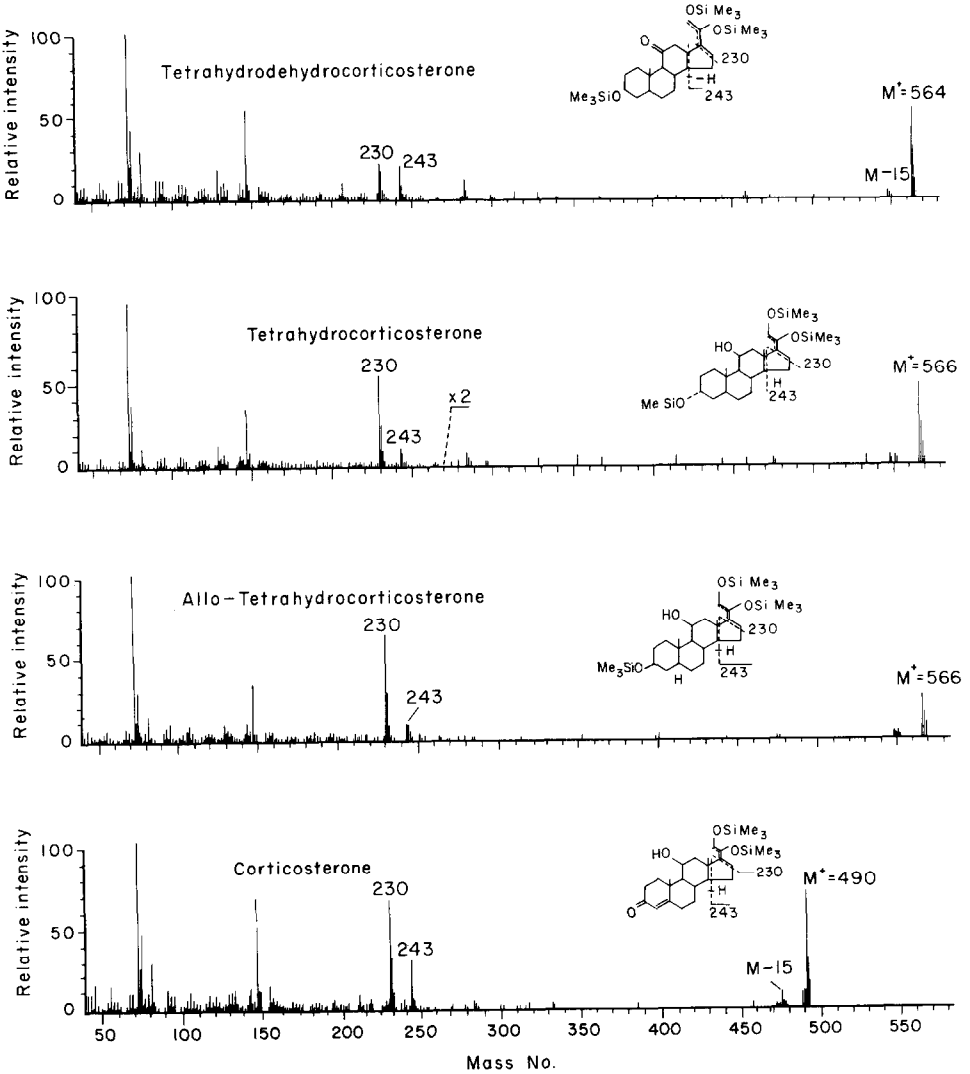

Fig. 5. Mass spectra of the THA, THB, α-THB and corticosterone derivatives.

($M^+ - 90 - 43$, $M^+ - 90 - 70$, $M^+ - 90 - 85$) corresponded to the cleavage of the unreacted lateral chain and of the unreacted lateral chain plus two and three carbon atoms of ring D, respectively. This indicated that only the 3-hydroxyl group was silylated. On the contrary, when there was an hydroxyl group at position 21, the 20-keto group reacted. In fact, the mass spectra of the derivatives of THA ($M^+ = 564$), THB and allo-THB ($M^+ = 566$) (Fig. 5) indicated that three TMS groups were present in the molecule.

The ions at m/e 230 and 243 which appeared in the mass spectra of these compounds were probably fragments containing the side chain holding two TMS groups plus two and three carbon atoms of ring D, respectively. In any case, this data demonstrated that the third silylation involved the enolic form of the 20-keto group. A similar behaviour was noticed for corticosterone (Fig. 5) $M^+ = 490$) as well. In this case, however, the 3-keto in addition to the 11β-hydroxyl group remained unreactive.

Enolization of the 20-ketone and subsequent derivatization also took place with corticosteroids bearing a dihydroxyacetone side-chain as already reported [4]. The mass spectra of THE, THS and THF showed the presence of a very intense peak at m/e 331 corresponding to the loss of the lateral chain containing three TMS groups plus two carbon atoms of ring D[4]. This peak was also present in the mass spectra of similarly treated cortisone and cortisol (Fig. 6); in this instance, the molecular ions (m/e 576 and 578 respectively) showed that only 3 TMS groups were present and confirmed that the keto groups at 3 and 11 were unreactive under the described conditions.

Cortol, β-cortol ($M^+ = 584$), cortolone and β-cortolone ($M^+ = 582$) (Fig. 7) gave rise to trisilylated derivatives. As observed by Aringer *et al.*[8] in the case of the tetra-TMS derivatives of cortol and β-cortol, the peaks at $M^+ - 133$ suggest that 20-hydroxyl remained unaffected, while the peaks at m/e 116 and 205 indicated the presence of an unreacted 17α-hydroxyl group.

This is consistent with the hypothesis that two derivatives were obtained from each compound (a 17,21-bis-TMS ether and a 20,21-bis-TMS ether) and that they have the same retention time.

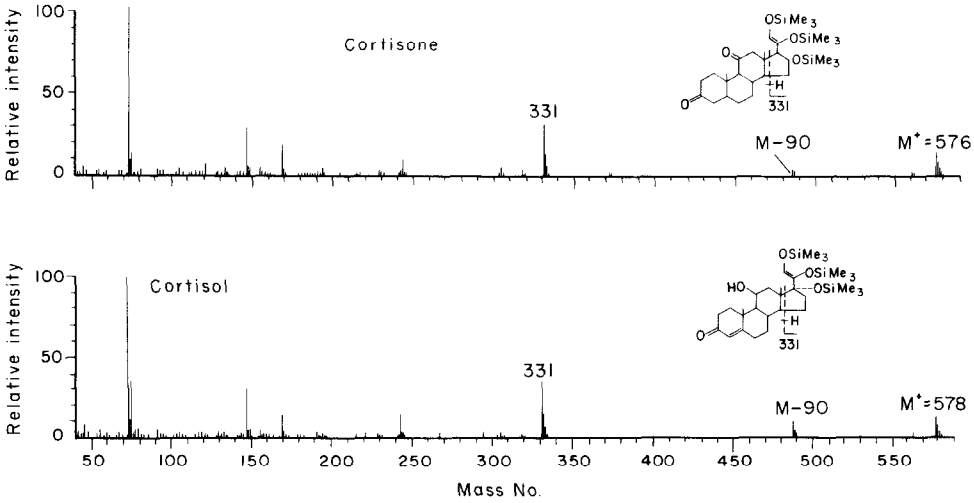

Fig. 6. Mass spectra of the cortisone and cortisol derivatives.

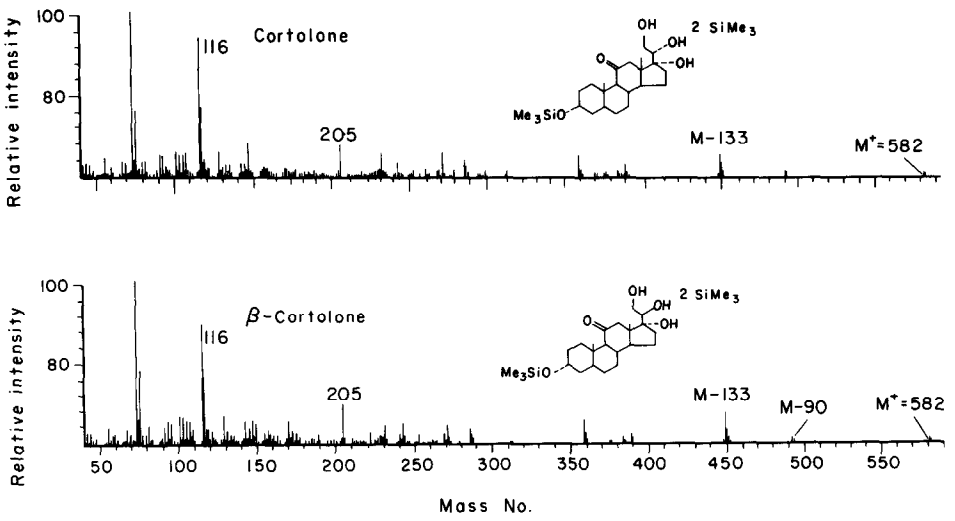

Fig. 7. Mass spectra of the cortolone and β-cortolone derivatives.

The TMS-enol-TMS derivatives appeared to be very convenient for gas chromatographic and gas chromatographic–mass spectrometric determination. A single reaction product from the most important steroid of biological interest was obtained in a quantitative and reproducible fashion. This reaction can be applied for the simultaneous determination of many urinary steroids in the study of reproductive physiology and of gonadal or adrenocortical functions.

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