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CHARACTERIZATION OF METABOLITES OF STEROID CONTRACEPTIVES BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

Metabolites isolated from human urine after oral administration of dimethisterone (6α-methyl-17α-(1-propynyl)-17β-hydroxy-4-androsten-3-one) and norethisterone (17α-ethynyl-17β-hydroxy-4-estren-3-one) were identified by gas chromatography and mass spectrometry. The major metabolite of dimethisterone is 6α-methyl-17α-(1-propynyl)-5β-androstane-3α, 17β-diol. The major urinary metabolites of norethisterone are 17α-ethynyl-5β-estrane-3α, 17β-diol and 17α-ethynyl-5α-estrane-3α, 17β-diol. The drug metabolites can be readily differentiated from urinary metabolites of endogenous steroids by both gas chromatographic retention data and mass spectrometry.

INTRODUCTION

STEROIDAL oral contraceptives are used extensively in the control of fertility. However, little information is available concerning the metabolism of certain of these drugs in humans. Investigations of the metabolism of orally and/or intravenously administered radioactive-labeled steroidal drugs have provided information on their rate of excretion and nature of certain conversion products [1–3]. The study of steroidal drug metabolites in humans using gas chromatography and mass spectrometry has furnished an additional means for determining the structure of the different metabolites. These methods are particularly suitable for the identification of individual components occurring in mixtures in low concentration. Human urinary metabolites of norgestrel (racemic 13β-ethyl-17α-ethynyl-17β-hydroxy-4-gonen-3-one) have been identified by mass spectrometry and other analytical techniques [4]. Norethynodrel (17α-ethynyl-17β-hydroxy-5(10)estren-3-one) was found to be metabolized to the 3α- and 3β-diols [5]. Brooks *et al.* [6] studied the metabolism of nilevar (17α-ethyl-17β-hydroxy-4-estren-3-one) using gas chromatography-mass spectrometry and found the urinary drug metabolites were readily recognized by fragmentation behavior and selective detection of characteristic ions.

In the present communication we report the identification of urinary metabolites of two progestational drugs, dimethisterone and norethisterone (norethindrone). Dimethisterone differs from the synthetic progestagens used in many preparations in that it is not a 19-nortestosterone derivative. It retains the C-10 methyl group and in addition carries a methyl group at C-6 and a propynyl group at C-17. Norethisterone, one of the earliest drugs used clinically and the progestational component of some currently used preparations, is in the 19-nortestosterone series and possesses an ethynyl substituent at C-17. The metabolism and recovery of radioactivity in various urinary fractions following the administra-

tion of labeled norethisterone to human subjects has been studied by various groups[1–3, 7]. Little or no metabolism of the ethynyl group was found to occur [2, 7]. Some investigators[8, 9] reported that norethisterone acetate is converted in humans to a small extent to estrogenic metabolites, primarily 17α-ethynylestradiol. However, a subsequent report[10] indicates that norethisterone is not converted to estrogens, but that phenolic compounds arise as artifacts during the analytical procedure. In one study[11] the main metabolites of norethisterone were reported to be 17α-ethynyl-17β-hydroxy-5α-estran-3-one and 17α-ethynyl-5α-estrane-3β, 17β-diol. The former compound is of interest as various groups reported that following the administration of labeled norethisterone approximately half of the extractable radioactivity is present in the ketonic fraction[2, 7].

EXPERIMENTAL

Reference compounds

Dimethisterone was provided by Dr. Gordon McKinney (Mead Johnson Research Center). Norethisterone was purchased from Sigma Chemical Co. The following steroids were obtained from Mann Research Labs.: 3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-17-one, and 3β-hydroxy-5-androsten-17-one. Dr. Paige Besch kindly supplied 17α-ethynyl-5β-estrane-3α, 17β-diol and 17α-ethynyl-5α-estrane-3α,17β-diol. 17α-Ethynyl-5β-estrane-3β, 17β-diol was obtained from Dr. Stephen Kraychy (G. D. Searle Co.).

17α-Ethynyl-17β-hydroxy-5α-estran-3-one was prepared by lithium-ammonia reduction of norethisterone[12].

17α-Ethynyl-5α-estrane-3β,17β-diol was prepared from 17α-ethynyl-17β-hydroxy-5α-estran-3-one by reduction with sodium borohydride in aqueous dioxane[12].

6α-Methyl-17α-propynyl-17β-hydroxy-5α-androstan-3-one was prepared by lithium-ammonia reduction of dimethisterone[12].

6α-Methyl-17α-propynyl-5α-androstane-3β, 17β-diol was obtained by reduction of the corresponding 3-ketone with sodium borohydride in dioxane.

6α-Methyl-17α-propynyl-5α-androstane-3α, 17β-diol was obtained by reduction of the 3-ketone by the Henbest method[13] (chloroiridic acid, trimethylphosphite, isopropanol and water).

For the preparation of 6α-methyl-17α-propynyl-5β-androstane-3α, 17β-diol, 40 mg of dimethisterone was dissolved in anhydrous ethanol (15 ml), cooled in an ice bath and treated with excess sodium for 1 hour. Extraction yielded a mixture of alcohols which were shown by gas chromatography and mass spectrometry to be the 6α-methyl-17α-propynyl-5α-androstane-3β,17β-diol (ca. 85%), the 6α-methyl-17α-propynyl-5α-androstane-3α,17β-diol (1·5%) and the 6α-methyl-17α-propynyl-5β-androstane-3α,17β-diol (11%). The latter compound was separated from its isomers by careful chromatography over neutral alumina (10 gm column) using a benzene-ether gradient. Its structure was confirmed by oxidation with chromium trioxide-pyridine-water[14] to 6α-methyl-17α-propynyl-17β-hydroxy-5β-androstan-3-one, which differed in gas chromatographic behavior from the corresponding 5α-3-one obtained previously. 6α-Methyl-17α-propynyl-5β-androstane-3α,17β-diol was regenerated from the 6α-methyl-17α-propynyl-17β-hydroxy-5β-androstan-3-one by treatment with sodium borohydride in aqueous dioxane. 6α-Methyl-17α-propynyl-5β-androstane-3β,17β-diol was

obtained by the Henbest reduction of the corresponding 5β-3-one[13].

The reference steroids were characterized by gas chromatographic retention data and mass spectrometry.

Metabolic studies

Oracon (consisting of 25 mg of dimethisterone and 0·5 mg of ethynyl estradiol) was administered to a normal adult female for a period of five days. In the norethisterone study 10 mg of norethisterone was orally administered for 2 days. Urine samples during the 24 h period were collected, diluted to a vol. of 2 l and stored at −15°C. A control sample of urine (no administered drug) was similarly collected and stored.

Aliquots of urine (250 ml) were subjected to enzymic hydrolysis at pH 4-5 (acetate buffer) with 4 ml of Glusulase* (Endo Labs) for 24 h at 37°C. The pH was adjusted to 5·5; an additional 4 ml of Glusulase was added and the hydrolysis was continued for another 24 h. The steroids were extracted with dichloromethane (2 × 500 ml), then with ethyl acetate (1 × 500 ml). The combined organic layers were concentrated to a vol. of about 150 ml and washed with three 50 ml portions of 5% sodium bicarbonate-10% sodium chloride solution and two 50 ml portions of 10% sodium chloride solution. The organic layer was dried over anhydrous magnesium sulfate and the solvents were removed (Rotovap).

Chromatographic analysis

Column chromatography was carried out on silicic acid[15] (Unisil, 200-325 mesh, Clarkson Chemical Co.). The urinary steroids were separated into the following 20 ml fractions: Fraction 1, 10% ethyl acetate in benzene; fraction 2, 27% ethyl acetate in benzene; fraction 3, 35% ethyl acetate in benzene; fraction 4, ethyl acetate; and fraction 5, methanol. The eluates were evaporated to dryness and the residues were transferred (methanol) to small screw-capped vials (Teflon liners) for derivative formation. Fraction 2 contained the steroidal drug metabolites.

A portion of fraction 2 was separated into ketone and non-ketonic fractions by means of Girard's reagent T[16]. The non-ketonic fraction was used for mass spectrometric analysis.

Formation of derivatives. Trimethylsilyl (TMSi) ethers of the steroids were prepared by dissolving the samples (200–300 μg) in 125 μl of *bis*(trimethylsilyl) acetamide (BSA) and 125 μl of trimethylsilylimidazole (TSIM) and heating for 2 h at 130°C. The O-methyloxime-trimethylsilyl (MO-TMSi) derivatives were prepared by the addition of 10·0 mg of dry methoxyamine hydrochloride in 0·5 ml of dry pyridine to the steroid sample. The mixture was allowed to stand overnight at room temperature. The solvent was evaporated (nitrogen stream) and the residue was dissolved in 125 μl of BSA and 125 μl of TSIM. After 2 h at 130°C the sample was analyzed by gas chromatography and mass spectrometry. Deuterated trimethylsilyl derivatives[17] were prepared using *bis*(trimethylsilyl)acetamide-d_{18} and trimethylsilylimidazole-d_9 (Merck Sharp and Dohme, Montreal, Canada).

Gas–liquid chromatography (GLC) was carried out using a Barber–Colman model 5000 instrument with a hydrogen flame detection system. Glass columns

*Each ml contains 179,500 units of glucuronidase and 47,000 units of sulfatase.

(12 ft × 4 mm W-tubes) were silanized and packed with 1% SE-30 and 1% OV-17 (Supelco, Inc.) on 100–120 mesh Gas Chrom P (Applied Science Labs.). Column packings were prepared according to the procedure described by Horning *et al.*[18]. Nitrogen was used as carrier gas with a flow rate of 30–40 ml/min. The injector block temperature was 260°C and the detector bath was at 280°C. The separations were carried out by temperature programming at a rate of 1°C per min from 180°C. Methylene unit (MU) values[19] were determined with *n*-alkanes as reference compounds under a temperature programmed rate of 1°C/min from 180°C.

Gas chromatography-mass spectrometry (GC-MS) was performed with an LKB 9000 instrument. Glass coiled columns (9 ft × 4 mm) of 1% SE-30 and 1% OV-17 on Gas Chrom P were used in the temperature programmed analyses. The ion source temperature was 250°C; the ionizing current was 60 μA; electron energy was 70 eV.

RESULTS AND DISCUSSION

Steroid metabolites

Steroidal drug metabolites isolated from human urine after oral administration of dimethisterone and norethisterone were found in fraction 2. Fraction 2 also contained the normal urinary metabolites 3α-hydroxy-5-androsten-17-one, androsterone, etiocholanolone, and dehydroepiandrosterone.

Gas–liquid chromatographic separation of the MO-TMSi derivatives of fraction 2 (Fig. 1) isolated after dimethisterone administration showed a peak which was not present in the corresponding fraction in the control urine. The mass spectrum of this compound (Fig. 2) as the di-TMSi derivative showed a molecular ion at *m/e* 488. The four stereoisomers of the alcohol were synthesized and the gas chromatographic retention data (Table 1) and mass spectra (Table 2) compared with that of the human metabolite, which was found to be 6α-methyl-17α-propynyl-5β-androstane-3α, 17β-diol.

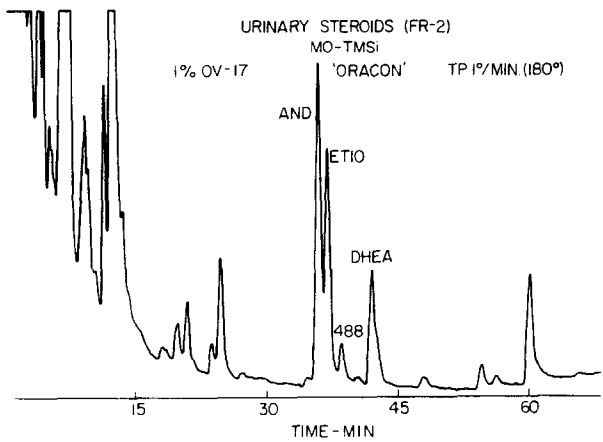

Fig. 1. Gas chromatographic analysis of urinary steroids (fraction 2), after oral administration of dimethisterone, as the MO-TMSi and TMSi derivatives. Labeled components are AND (androsterone), ETIO (etiocholanolone), DHEA (dehydroepiandrosterone) and 488 (dimethisterone metabolite). Column and conditions: 1% OV-17, 180–260° at 1°/min.

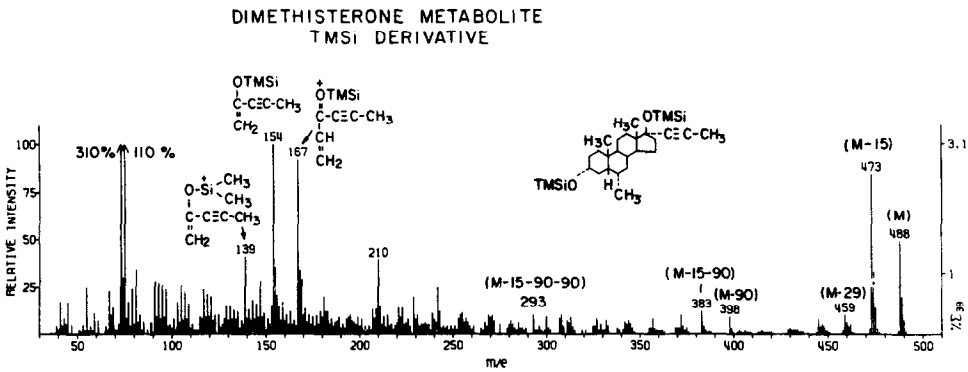

Fig. 2. Mass spectrum of the urinary metabolite of dimethisterone as TMSi derivative (peak labeled 488 in Fig. 1).

Table 1. Methylene unit values of reference steroids and drug metabolites

Compound	SE-30		OV-17	
	Free	TMSi	Free	TMSi
6α-methyl-17α-(1-propynyl)-17β-hydroxy-5α-androstan-3-one	26·92	----	31·62	----
6α-methyl-17α-(1-propynyl)-17β-hydroxy-5β-androstan-3-one	26·20	----	30·78	----
6α-methyl-17α-(1-propynyl)-5α-androstane-3α, 17β-diol	26·69	26·80	30·80	28·10
6α-methyl-17α-(1-propynyl)-5α-androstane-3β, 17β-diol	26·78	27·98	31·07	29·10
6α-methyl-17α-(1-propynyl)-5β-androstane-3α, 17β-diol	25·90	26·40	30·25	27·75
6α-methyl-17α-(1-propynyl)-5β-androstane-3β, 17β-diol	25·88	26·70	30·35	27·60
Dimethisterone metabolite	25·98	26·40	30·28	27·62
17α-ethynyl-17β-hydroxy-5α-estran-3-one	24·31	----	28·55	----
17α-ethynyl-5α-estrane-3α, 17β-diol	24·00	25·45	27·98	26·43
17α-ethynyl-5α-estrane-3β, 17β-diol	24·71	26·18	28·06	27·32
17α-ethynyl-5β-estrane-3α, 17β-diol	24·14	25·87	28·24	26·95
17α-ethynyl-5β-estrane-3β, 17β-diol	24·13	25·85	28·15	26·77
Norethisterone metabolite (major)	24·14	25·88	28·23	26·98
Norethisterone metabolite (minor)	----	25·50	----	26·46

Gas chromatographic analysis of fraction 2(Fig. 3) isolated from the urinary extract following norethisterone administration showed the presence of two peaks which were not present in the control sample. Mass spectra obtained from both peaks gave molecular ions at *m/e* 446. The mass spectrum of the major gas chromatographic peak is shown in Fig. 4. Comparison with reference steroids (Tables 1 and 2) established its structure to be 17α-ethynyl-5β-estrane-3α, 17β-diol. Comparison with reference steroids showed the minor urinary metabolite of norethisterone to be 17α-ethynyl-5*a*-estrane-3*a*, 17β-diol. The ratio of the two metabolites of norethisterone, 17*a*-ethynyl-5β-estrane-3*a*, 17β-diol to 17α-ethynyl-5α-estrane-3α,17β-diol, is a little more than 4:1. Previous studies on the metabolism of 19-nortestosterone[20] following oral administration indicated that the ratio of *cis* (5β) to *trans* (5α) urinary metabolites was approximately 1:2. It has been reported[21] that the major urinary products isolated after administration of 1α-methyl-3α-hydroxy-5β-estran-17-one and 1α-methyl-3α-hydroxy-5α-estran-17-one occur in ratios of approximately 10:1. Thus it

Table 2. Mass-to-charge ratios and relative intensities of major ions in the mass spectra of the di-TMSi derivatives of 6α-methyl-17α-(1-propynyl)-5α/5β-androstane-3α/3β, 17β-diol and 17α-ethynyl-5α/5β-estrane-3α/3β, 17β-diol.

Compound	M	M-CH_3	M-C_2H_3	M-TMSiOH	M-TMSiOH-CH_3	Ion A[f]	Ion B	Ion C	Ion D
5α-3α-I[a]	488(34)[c]	473(47)	459(7)	398(4)	383(5)	210(33)	167(89)	154(100)	139(39)
5α-3β-I	488(53)	473(86)	459(11)	398(4)	383(5)	210(24)	167(100)	154(86)	139(34)
5β-3β-I	488(39)	473(64)	459(8)	398(11)	383(12)	210(39)	167(100)	154(87)	139(39)
5β-3α-I	488(44)	473(81)	459(10)	398(7)	383(11)	210(35)	167(98)	154(100)	139(50)
5α-3β-I-d_{18}[d]	506(35)	491(27)	477(7)	407(3)	392(4)	219(23)	176(100)	163(79)	145(32)
Dimethisterone metabolite	488(49)	473(84)	459(10)	398(9)	383(12)	210(39)	167(92)	154(100)	139(41)
5α-3α-II[b]	446(27)	431(100)	417(10)	356(5)	341(7)	196(18)	153(14)	140(23)	125(13)
5α-3β-II	446(27)	431(100)	417(11)	356(5)	341(7)	196(15)	153(14)	140(22)	125(11)
5β-3α-II	446(20)	431(100)	417(8)	356(8)	341(13)	196(24)	153(15)	140(26)	125(14)
5β-3α-II-d_{18}[e]	464(48)	449(100)	435(12)	365(13)	350(11)	205(37)	162(26)	149(36)	131(28)
Norethisterone major metabolite	446(20)	431(100)	417(7)	356(8)	341(11)	196(23)	153(15)	140(24)	125(13)
Norethisterone minor metabolite	446(30)	431(100)	417(11)	356(8)	341(14)	196(24)	153(17)	140(32)	125(17)

[a] I = 6α-methyl-17α-(1-propynyl)-5α/5β-androstane-3α/3β-diol *bis*(trimethylsilyl) ether.
[b] II = 17α-ethynyl-5α/5β-estrane-3α/3β,17β-diol *bis*(trimethylsilyl) ether.
[c] m/e (relative intensity).
[d] 6α-methyl-17α-propynyl-5α-androstane-3β, 17β-diol *bis*(trimethylsilyl-d_9) ether.
[e] 17α-ethynyl-5β-estrane-3α, 17β-diol *bis*(trimethylsilyl-d_9) ether.
[f] Chart A.

appears that the ratio of the two isomers formed is dependent upon the substituents located on the steroid nucleus.

Mass spectrometry

The mass spectra of the four tetrahydro derivatives of dimethisterone as the TMSi ethers showed various intensity differences (Table 2). The isolated metabolite was found to closely correspond to the 5β-3α isomer. The ions at *m/e* 488 (M), 473 (M-CH_3), 398 (M-TMSiOH), 383 (M-CH_3-TMSiOH) and 293 (M-CH_3-TMSiOH-TMSiOH) of the metabolite establish the molecular weight. The presence of the unaltered D-ring with its substituents is shown by peaks at *m/e* 210, 167, 154, and 139. Plausible routes of formation of these ions are shown in Chart A ($R_1 = CH_3$, $R_2 = CH_3$, $R_3 = CH_3$). This type of fragmentation [6] is

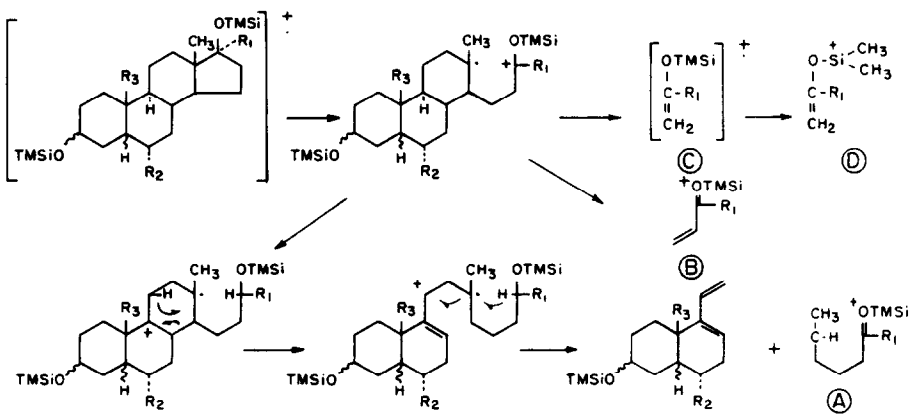

Chart A

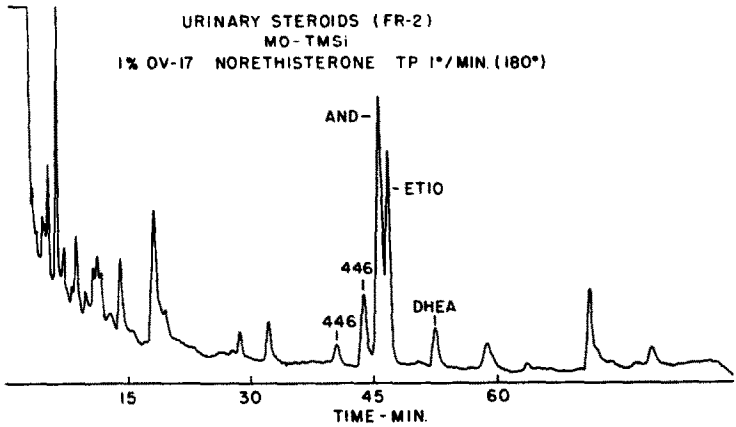

Fig. 3. Gas chromatographic analysis of the urinary steroids (fraction 2) after oral administration of norethisterone as the MO-TMSi and TMSi derivatives. Labeled components are AND (androsterone), ETIO (etiocholanolone), DHEA (dehydroepiandrosterone) and 446 (norethisterone metabolites). Column and conditions same as for Fig. 1.

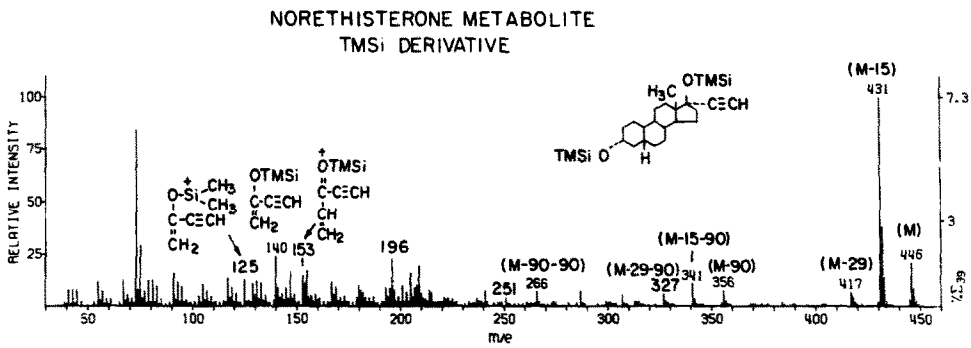

Fig. 4. Mass spectrum of the major urinary metabolite of norethisterone as the TMSi derivative.

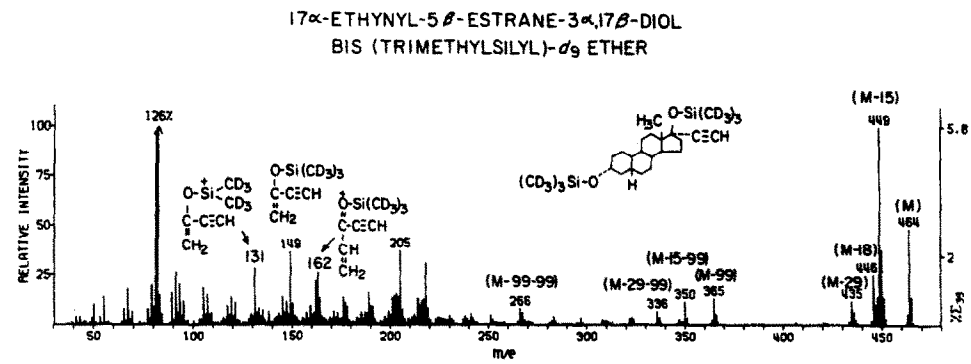

Fig. 5. Mass spectrum of the deuterated TMSi derivative of 17α-ethynyl-5β-estrane-3α, 17β-diol.

valuable in structural analysis of the many 17α-substituted 17β-hydroxysteroids. These ions, also found in the TMSi derivative of dimethisterone itself, form a considerable proportion of the total ion current.

The mass spectra of the alcohols derived from norethisterone showed only minor intensity differences (Table 2). The fragmentation is similar to that of the

dimethisterone derivatives (Chart A, $R_1 = H$, $R_2 = H$, $R_3 = H$). As shown in Fig. 5 and Table 2 the structures of these ions are supported by their shift on preparation of the TMSi derivatives with the deuterated reagents *bis*(trimethylsilyl)acetamide-d_{18} and trimethylsilylimidazole-d_9.

A notable feature of the spectra of these TMSi derivatives is the loss of CH_3 primarily from the angular positions (compare the spectra of the deuterated and undeuterated compounds in Table 2 and Figs. 4 and 5). This is in contrast to the less prominent loss of the angular substituents observed by DeJongh *et al.* [4] for the underivatized compounds (norgestrel, norethisterone, and *dl*-13β-ethyl-17α-ethynyl-5β-gonane-3α, 17β-diol). The small but prominent peak at M-29 and the related ion at M-29-90 in Figs. 4 and 5 must be derived from the steroid skeleton and do not indicate the presence of an ethyl substituent.

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