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ANALYSIS OF 5α-ANDROSTANEDIOL MONOACETATES BY THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

Selective acetylation of the 3α-, 3β- or 17β-hydroxyl group in 5α-androstane-3α,17β- and -3β,17β-diol has been suspected to occur in male target organs. This work describes the synthesis of four 5α-androstanediol monoacetates and their separation either by thin-layer or gas–liquid chromatography. Complete separation of the four isomers was achieved on thin layers of alumina. Two liquid phases were found to resolve these steroids after their conversion into the trimethylsilyl derivatives. Identification was completed by mass spectrometry.

INTRODUCTION

The presence of conjugated 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol in human urine has been reported by us[1,2]. The labelled epimers are also formed as products of the *in vivo* and *in vitro* metabolism of [4-^{14}C]testosterone in the human prostate with benign hypertrophy[3]. These metabolites play a significant role in the mode of action of the hormone at the level of some male target organs[4,5].

Work in connection with the identification of a radioactive product with the properties of a hydroxysteroid monoacetate led us to consider the possibility that selective acetylation of the 3α-, 3β- or 17β-hydroxyl group of the above androstanediols had occurred. Accordingly, we prepared the required reference compounds, established conditions for the resolution of mixtures of the epimers and sought criteria for identification. In this paper we report the mobilities of these monoacetates on thin-layer chromatography (TLC) and retention indices on gas–liquid chromatography (GLC) and present their mass spectra.

MATERIALS AND METHODS

Synthesis of 17β-acetoxy-5α-androstane-3β,17β-diol (A)

17β-Acetoxy-5α-androstan-3-one was obtained by acetylation of 17β-hydroxy-5α-androstan-3-one (Roussel-Uclaf, Paris, France). The acetate was crystallized once from *n*-hexane and its purity checked by TLC. Reduction of the 3-oxo-steroid acetate dissolved in methanol was carried out with potassium borohydride in 2 h. TLC showed

that the 17β-acetoxy-5α-androstane-3β,17β-diol obtained was contaminated with the 3α-epimer. Successive crystallizations from methanol (twice) and methanol–*n*-hexane (three times) did not eliminate the 3α-contaminant completely. Pure 17β-acetoxy-5α-androstane-3β,17β-diol was obtained by preparative TLC and the eluted compound was crystallized from *n*-hexane. The crystals (m.p. 148–149°) gave a single zone on TLC and a single peak on GLC. Characteristic absorption bands were obtained with a Beckman (Fullerton, Calif., U.S.A.) IR-18A infrared spectrometer when crystals dissolved in carbon disulphide were analyzed ($\nu_{OH} = 3610\ cm^{-1}$; $\nu_{C=O} = 1740\ cm^{-1}$; $\nu_{C-O-C} = 1250$, 1032 and $1050\ cm^{-1}$).

Synthesis of 17β-acetoxy-5α-androstane-3α,17β-diol (B)

The tetrahydropyranyl ether of androsterone was obtained by treating androsterone (E. Merck, Darmstadt, G.F.R.) for 90 min with distilled 2,3-dihydropyran in the presence of *p*-toluenesulphonic acid. Specific reduction of the 17-keto-group in androsterone tetrahydropyranyl ether to a 17β-hydroxy-group was carried out by treatment with lithium tri-*tert*.-butoxyaluminium hydride (Merck) for 32 h at room temperature. The reduced product was acetylated and crystallized from methanol. Partial regeneration of the 3α-hydroxy-group was obtained by adding diluted perchloric acid to the crystals dissolved in acetone. 17β-Acetoxy-5α-androstane-3α,17β-diol was obtained and separated from the parent tetrahydropyranyl ether by partition between *n*-hexane and 80% methanol. The dihydroxy-steroid monoacetate was crystallized three times from methanol. The crystals (m.p. 191–192°) gave a single zone on TLC, a single peak on GLC and characteristic absorption bands on IR spectrometry ($\nu_{OH} = 3440\ cm^{-1}$; $\nu_{C=O} = 1700\ cm^{-1}$; $\nu_{C-O-C} = 1270$ and $1028\ cm^{-1}$, in potassium bromide micro-pellets).

Synthesis of 3β-acetoxy-5α-androstane-3β,17β-diol (C)

3β-Acetoxy-5α-androstan-17-one was obtained by acetylation of 3β-hydroxy-5α-androstan-17-one (Merck). The acetate was crystallized once from *n*-hexane and its purity checked by TLC. Specific reduction of the 17-oxo-steroid acetate dissolved in *tert*.-butanol to the 17β-hydroxy-derivative was carried out by reaction for 54 h at room temperature with lithium tri-*tert*.-butoxyaluminium hydride. The reaction mixture was checked by TLC and the resulting 3β-acetoxy-5α-androstane-3β,17β-diol was purified by column chromatography on alumina. The purified steroid was crystallized twice from methanol. The crystals (m.p. 108–109°) gave a single zone on TLC, a single peak on GLC and characteristic absorption bands on IR spectrometry ($\nu_{OH} = 3620\ cm^{-1}$; $\nu_{C=O} = 1745\ cm^{-1}$; $\nu_{C-O-C} = 1245$ and $1025\ cm^{-1}$ for crystals dissolved in *n*-hexane).

Synthesis of 3α-acetoxy-5α-androstane-3α,17β-diol (D)

3α-Acetoxy-5α-androstan-17-one was obtained from acetylation of androsterone (Sigma). The acetate was crystallized once from methanol and its purity checked by TLC. Specific reduction of the 17-oxo-steroid acetate dissolved in *tert*.-butanol to the 17β-hydroxy-derivative was carried out in 26 h at room temperature in the presence of lithium tri-*tert*.-butoxyaluminium hydride. The resulting 3α-acetoxy-5α-androstane-3α,17β-diol was crystallized twice from *n*-hexane from the reaction mixture extract and further purified by preparative TLC. The eluate was crystallized

once again from *n*-hexane. The crystals (m.p. 183–184°) gave a single zone on TLC, a single peak on GLC and characteristic absorption bands on IR spectrometry ($\nu_{OH} = 3470\ cm^{-1}$; $\nu_{C=O} = 1705\ cm^{-1}$; $\nu_{C-O-C} = 1270$, 1260 and $1032\ cm^{-1}$ in potassium bromide micro-pellets).

Derivative formation

All derivatives were prepared from 50 μg of the free steroids. Reaction mixtures were evaporated to dryness at 60° under nitrogen, the dry residues were dissolved in 200 μl of carbon disulphide and 2-μl portions of the solution injected into the GLC column.

The trimethylsilyl (TMSi) derivatives were prepared in 30 min at 60° after addition to the dry steroids of 100 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 10 μl of pyridine.

The heptafluorobutyrate (HFB) derivatives were prepared by adding to the dry steroids 1 ml of heptafluorobutyric anhydride–tetrahydrofuran–*n*-hexane (1:1:10) and heating the mixture at 60° for 1 h.

The trifluoroacetyl (TFA) derivatives were obtained by reaction of the dry steroids with 200 μl of trifluoroacetic anhydride–tetrahydrofuran (1:10) at 60° for 1 h.

Thin-layer chromatography

Thin-layer plates (20 × 20 cm) were coated with silica gel GF_{254} (Merck) layers of 0.5 mm thickness and dried at room temperature, and plates pre-coated with aluminium oxide (Merck) were also used; in both instances, the plates were activated for 30 min at 120° before use. The solvent mixtures were not allowed to saturate the tanks, a single development being carried out immediately after pouring the freshly prepared mixture into the tank. Details of the solvent mixtures used and steroids tested are given in Table I. Steroids were rendered visible on the chromatograms by spraying with sulphuric acid–methanol (7:3) and heating at 120° for 15 min.

Gas–liquid chromatography

Carlo Erba (Milan, Italy) GI-452 and Pye Unicam (Cambridge, Great Britain) 104-84 chromatographs were used, both equipped with flame ionization detectors operated at 250°. Retention times were measured with an Infotronics (Boulder, Colo., U.S.A.) CRS-104 electronic integrator coupled with a Servo-Riter (Texas Instr., Houston, Texas, U.S.A.) recorder, and retention indices were calculated and expressed according to the method of Kováts[6]. Silanized glass columns were packed with different stationary phases on Gas-Chrom Q (100–120 mesh) and nitrogen was used as the carrier gas at a flow-rate of 40 ml/min. Operating conditions are given in Table II.

Gas–liquid chromatography–mass spectrometry (GC–MS)

A double-beam AEI (Manchester, Great Britain) MS-30 mass spectrometer coupled by a silicone membrane separator to a Pye Unicam 104 gas chromatograph was used. The analysis of free compounds was performed with a 3 m × 3 mm O.D. glass column packed with 1.63% QF-1 and operated at 210°. Derivatives were injected into a 2.5 m × 3 mm O.D. glass column packed with 1.55% OV-225 and operated at 213°. In all instances the helium gas flow-rate was 30 ml/min. The temperatures of the molecular separator, introduction line and ion source were 200°, 210° and 200°,

respectively. The energy of the bombarding electrons was 24 eV and the ionizing current was 300 μA. Mass spectra were taken with a scan speed of 3 sec per decade at a resolution of 1000.

RESULTS AND DISCUSSION

Thin-layer chromatography

The R_F values in different TLC systems of the four 5α-androstanediol monoacetates are given in Table I, together with those of androst-4-ene-3,17-dione and 17β-hydroxy-5α-androstan-3-one, which have similar chromatographic mobilities and may be found in biological extracts.

TABLE I

R_F VALUES OBTAINED BY THIN-LAYER CHROMATOGRAPHY OF THE FOUR 5α-ANDROSTANEDIOL MONOACETATES (A, B, C AND D) IN DIFFERENT SOLVENT MIXTURES

A single development was carried out on the following solvent mixtures: I, chloroform–acetone (9:1); II, benzene–ethanol (9:1); III, diisopropyl ether–acetone (4:1); IV, chloroform–methanol–water (118:12:1); V, cyclohexane–ethyl acetate (1:1); VI, benzene–ethyl acetate (1:1); VII, dichloromethane–diethyl ether (9:1); VIII, benzene–ethanol (97:3).

Monoacetate	*Thin layer*								
	GF_{254}	GF_{254}	GF_{254}	GF_{254}	GF_{254}	GF_{254}	*Alumina G*	*Alumina G*	*Alumina G*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*I*
3β-OH,17β-OAc-5α-androstane (A)	0.53	0.25	0.57	0.54	0.62	0.55	0.36	0.33	0.57
3α-OH,17β-OAc-5α-androstane (B)	0.68	0.30	0.67	0.56	0.74	0.64	0.47	0.42	0.75
3β-OAc,17β-OH-5α-androstane (C)	0.72	0.29	0.69	0.55	0.74	0.64	0.55	0.41	0.66
3α-OAc,17β-OH-5α-androstane (D)	0.61	0.29	0.70	0.56	0.75	0.64	0.61	0.44	0.77
Androst-4-ene-3,17-dione	0.72	0.33	0.51	0.46	0.51	0.58	0.68	0.54	0.83
17β-OH-5α-androstan-3-one	0.48	0.27	0.59	0.48	0.61	0.53	0.51	0.39	0.74

In all instances, A has the lowest R_F value and can easily be separated from the other three isomers. None of the solvent systems used could resolve isomers B, C and D on layers of silica gel GF_{254}.

Alumina G was used successfully for the separation of the four isomers, which was accomplished with one development in dichloromethane–diethyl ether (9:1, v/v). The use of manufactured alumina thin-layer plates (Merck) helped to obtain fairly reproducible chromatograms. Nevertheless, a standard activation of thin layers of alumina was difficult to obtain and higher R_F values were observed when excessively deactivated plates were used.

Gas–liquid chromatography

In all systems, the 3α-oxygenated steroids (axial configuration) yielded lower retention times than the 3β-epimers with an equatorial configuration (see Table II).

The greater flatness of the latter molecule may favour interactions with the liquid phases and explain the differences in mobilities. In all derivatives, the 17β-acetoxy-groups were less polar than the 3α-acetoxy-substituents, and isomers B and D were therefore easily separated on polar and non-polar liquid phases. In contrast, isomers A and C were separated only as TFA derivatives on QF-1 and as TMSi derivatives on OV-225 and SILAR-5-CP phases. The four monoacetoxy-5α-androstanediol TMSi derivatives were clearly separated on OV-225 and SILAR-5-CP phases (see Fig. 1). The best resolution was obtained with the SILAR-5-CP liquid phase, which has recently been made available by Applied Science Lab. (State College, Pa., U.S.A.) as the most stable version of the frequently used polar steroid phases. On OV-225, columns with at least 2600 theorical plates were necessary in order to obtain an acceptable peak resolution ($R = 1.15$) for isomers A and C.

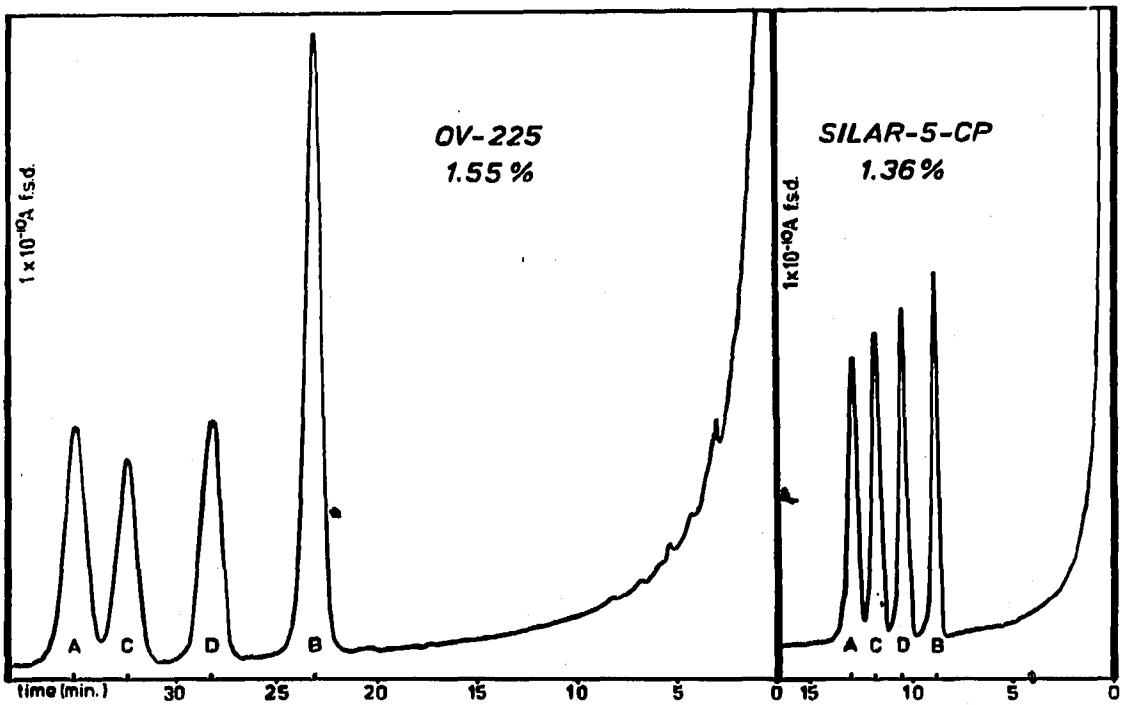

Fig. 1. Separation of the four 5α-androstanediol monoacetates (TMSi derivatives) by GLC on a 2.1 m × 4 mm column packed with 1.55% OV-225 on Gas-Chrom Q (100–120 mesh) and on a 2.1 m × 4 mm column packed with 1.36% SILAR-5-CP on the same support. A, B, C and D correspond to the formulae given in Fig. 2.

Mass spectra of free 5α-androstanediol monoacetates

The four mass spectra (see Fig. 2) showed a very small molecular peak at $m/e = 334$. From the fragmentation pattern of these compounds, the position of the acetoxy-group could be determined in each instance.

Only molecules with a 17β-acetoxy-group gave fragments at $m/e = 301$ (M−18−15), $m/e = 262$ (M−54−18) and $m/e = 165$ and 132. Furthermore, an intense

TABLE II

RETENTION INDICES OF 5α-ANDROSTANEDIOL MONOACETATES ON DIFFERENT LIQUID PHASES

A = 17β-acetoxy-5α-androstane-3β,17β-diol; B = 17β-acetoxy-5α-androstane-3α,17β-diol; C = 3β-acetoxy-5α-androstane-3β,17β-diol; D = 3α-acetoxy-5α-androstane-3α,17β-diol.

Monoacetate	*Dexsil, 1.32%, 2 m × 3 mm column, 240°*	*OV-7, 1.55%, 3.6 m × 3 mm column, 240°*	*QF-1, 1.63%, 3 m × 3 mm column, 210°*	*SP-2250, 1.0%, 1.5 m × 4 mm column, 220°*	*Hi-Eff 8 BP, 1.25%, 2.7 m × 4 mm column, 220°*	*OV-225, 1.55%, 2.1 m × 4 mm column, 208°*	*SILAR-5-CP, 1.36%, 2.1 m × 4 mm column, 220°*
A Free form	–	–	3320	2940	–	–	–
TMSi derivative	2780	3255	3145	2880	3180	3185	3368
TFA derivative	2690	2695	3320	2550	3170	3225	3340
HFB derivative	2700	2700	3360	2735	3045	3140	3255
B Free form	2760	–	3275	2935	–	–	–
TMSi derivative	2690	2945	3035	2785	3020	3055	3220
TFA derivative	2620	2620	3240	2670	3070	3160	3340
HFB derivative	2580	2585	3250	2615	2890	3010	3155
C Free form	2775	–	3310	2955	–	–	–
TMSi derivative	2785	3255	3135	2875	3175	3160	3330
TFA derivative	2690	2695	3295	2550	3165	3220	3390
HFB derivative	2700	2705	3350	2735	3065	3140	3250
D Free form	–	–	3250	2910	–	–	–
TMSi derivative	2755	3120	3090	2835	3100	3115	3288
TFA derivative	2655	2645	3240	2780	3085	3155	3345
HFB derivative	2660	2650	3300	2690	2980	3040	3200

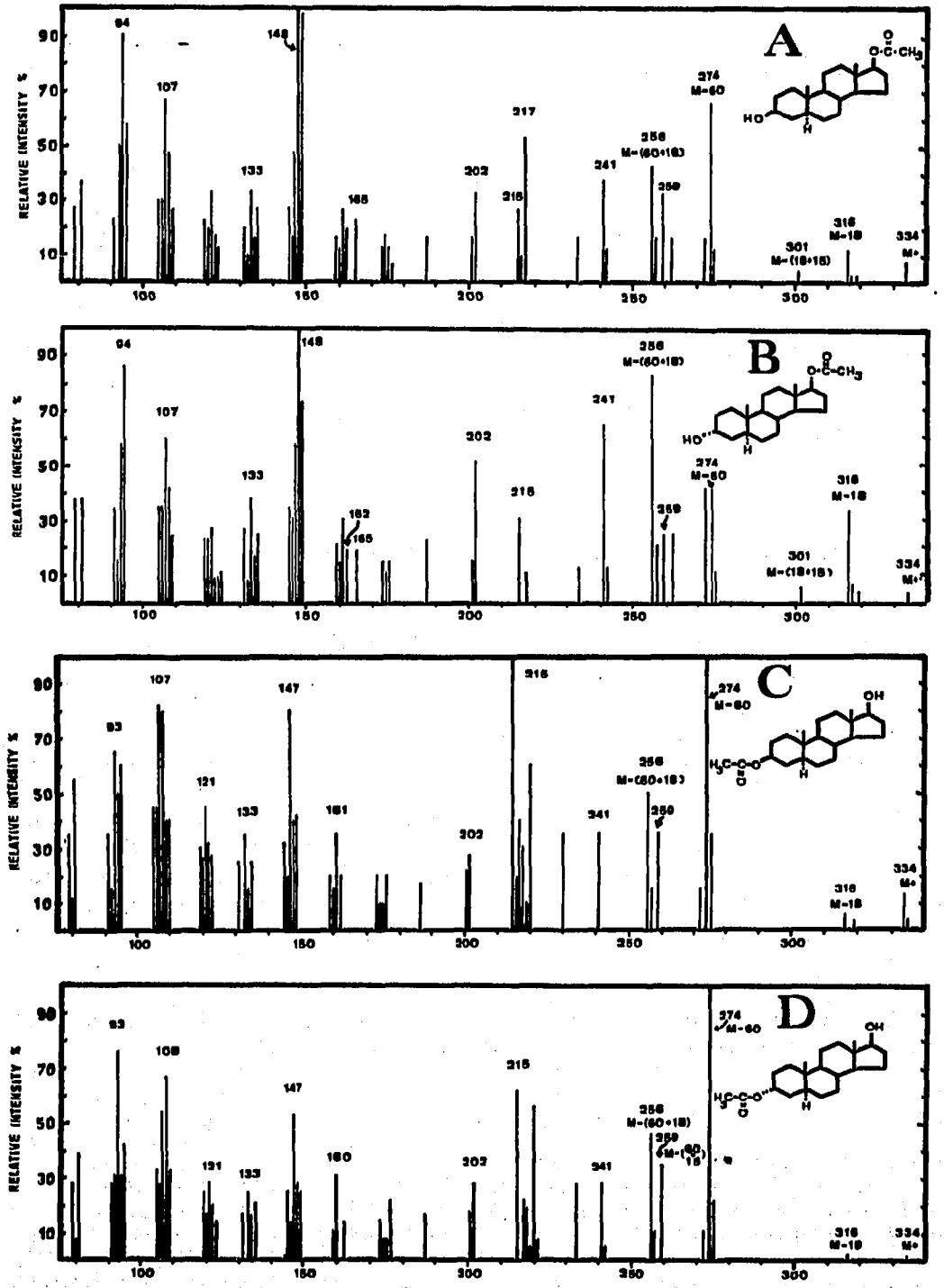

Fig. 2. Relative intensities of the principal fragments in the mass spectrometry of 5α-androstanediol monoacetates.

base peak at $m/e = 148$ characterized these molecules. In contrast, 3ξ-acetylated molecules gave a major fragment at $m/e = 274$ (M − 60), corresponding to the loss of acetic acid. In addition, molecules with a 3ξ-acetoxy-group were alone in giving fragment ions at $m/e = 220$ and $m/e = 218$, corresponding to the loss of fragment 114 (60 + 54) and 116 (60 + 56), respectively.

Mass spectra of 5α-androstanediol monoacetates derivatives

In all instances except the TMSi derivatives, the molecular and the M−15 ions were absent. Loss of 90, 60, 114 and 214 mass units corresponded to the loss of trimethylsilanol and acetic, trifluoroacetic and heptafluorobutyric acids, respectively.

TMSi Derivatives (see Table III). Structural information was provided by fragments at $m/e = 316$ (M−90), $m/e = 256$ (M−90−60), $m/e = 241$ (M−90−60−15), $m/e = 217$ (M−129−60) and $m/e = 215$ (M−131−60). The fragment at $m/e = 129$ was the base peak in the mass spectra of 3ξ-acetylated molecules and corresponded to the D-ring with the trimethylsiloxy-group[7,8]. This peak at $m/e = 129$ was much smaller in the mass spectra of 17β-acetylated molecules, thus allowing the determination of the position of the acetoxy-group[9].

TABLE III

RELATIVE INTENSITIES OF PRINCIPAL FRAGMENTS OF 5α-ANDROSTANEDIOL MONOACETATES (TMSi DERIVATIVES) IN MASS SPECTROMETRY

Derivative	*m/e*													
	406	*391*	*316*	*256*	*241*	*217*	*215*	*149*	*148*	*147*	*133*	*129*	*116*	*107*
A: 3β-OH,17β-OAc-5α-Androstane	6	100	17	9	8	29	6	9	9	14	9	10	0	25
B: 3α-OH,17β-OAc-5α-Androstane	3	0	100	33	22	52	14	20	17	23	10	15	0	28
C: 3β-OAc,17β-OH-5α-Androstane	4	10	19	15	24	7	14	24	16	13	18	100	25	17
D: 3α-OAc,17β-OH-5α-Androstane	4	0	9	29	28	14	26	15	17	14	16	100	26	15

TFA Derivatives (see Table IV). Molecules with a 17β-acetoxy-group gave the base peak at $m/e = 149$. This fragment corresponded to fragmentation of the androstane skeleton[7,10] and was absent in the mass spectra of 3ξ-acetylated molecules. Fragments at $m/e = 370$ (M−60) and $m/e = 316$ (M−69−54) were very important for these last compounds.

TABLE IV

RELATIVE INTENSITIES OF PRINCIPAL FRAGMENTS OF 5α-ANDROSTANEDIOL MONOACETATES (TFA DERIVATIVES) IN MASS SPECTROMETRY

Derivative	*m/e*												
	430	*370*	*355*	*329*	*316*	*256*	*241*	*215*	*149*	*148*	*147*	*109*	*108*
A: 3β-OH,17β-OAc-5α-Androstane	3	58	39	22	6	10	19	12	100	49	29	19	24
B: 3α-OH,17β-OAc-5α-Androstane	<1	46	32	20	25	13	25	16	100	42	25	18	26
C: 3β-OAc,17β-OH-5α-Androstane	<1	94	44	4	100	12	25	24	0	15	18	41	85
D: 3α-OAc,17β-OH-5α-Androstane	<1	100	40	10	86	10	20	19	4	9	23	44	8

HFB Derivatives (see Table V). Fragments were present at $m/e = 470$ (M−60), $m/e = 455$ (M−60−15), $m/e = 256$ (M−60−214), $m/e = 241$ (M−60−214−15)

TABLE V

RELATIVE INTENSITIES OF PRINCIPAL FRAGMENTS OF 5α-ANDROSTANEDIOL MONOACETATES (HFB DERIVATIVES) IN MASS SPECTROMETRY

Derivative	*m/e*													
	530	*470*	*455*	*416*	*384*	*256*	*241*	*215*	*161*	*149*	*147*	*109*	*108*	*107*
A: 3β-OH,17β-OAc-5α-Androstane	<1	71	32	0	16	9	12	10	14	100	25	12	29	43
B: 3α-OH,17β-OAc-5α-Androstane	<1	46	28	0	0	28	36	28	32	100	53	36	46	82
C: 3β-OAc,17β-OH-5α-Androstane	<1	92	40	100	28	11	22	28	10	8	30	40	80	40
D: 3α-OAc,17β-OH-5α-Androstane	<1	100	45	90	0	10	21	31	13	8	36	6	50	91

and $m/e = 215$ (M−60−255). As in TFA derivatives, the 17β-acetylated molecules gave the base peak at $m/e = 149$ and the 3ξ-acetylated compounds gave important fragments at m/e = M−60 and m/e = M−60−54. This last fragment might come from the A-ring with an acetyl group by a retro-Diels–Alder mechanism[11].

CONCLUSION

Both GLC and TLC permit the clear separation of the four 5α-androstanediol monoacetates. A combination of these techniques may prove to be useful when these compounds are to be isolated and purified from tissue extracts.

Identification by mass spectrometry is possible because characteristic fragmentation has been obtained for each 5α-androstanediol monoacetate.

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