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SEPARATION OF URINARY ANDROSTANEDIOL AND PREGNANEDIOL ISOMERS BY A COMBINED GAS–LIQUID CHROMATOGRAPHY–THIN-LAYER CHROMATOGRAPHY METHOD

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SUMMARY

Gas–liquid (GLC) and thin-layer chromatography (TLC) were used for the analysis of urinary C_{19} and C_{21} dihydroxysteroids. The behaviour of these steroids was studied systematically on seven stationary phases (SE-30, OV-1, OV-17, QF-1, XE-60, NGS and Hi-Eff 8 BP) and with different solvent systems on silica gel thin layers. The retention indices of the free steroids and their acetate, trimethylsilyl ether, trifluoroacetate, heptafluorobutyrate and chloromethyldimethylsilyl ether derivatives were determined.

Several combinations of TLC and GLC are proposed for the complete separation and identification of androstanediols and pregnanediols in biological fluids.

INTRODUCTION

In our attempts to develop a systematic method of analysis for C_{19} and C_{21} dihydroxysteroids[1,2], the exclusive use of gas–liquid chromatography (GLC) did not give satisfactory results. Natural mixtures of steroids often contain many isomers that are difficult to separate. Separations based on molecular weight and steric configuration are investigated on polar and non-polar liquid phases. The retention indices are expressed as steroid number (*SN*) according to VandenHeuvel and Horning[3]. Further, the analysis of structure–chromatography relationships is carried out using the "group number" (ΔSN) concept introduced by Feher and Bodrogi[4].

Separations largely dependent on differences in polarities are obtained by thin-layer chromatography (TLC) on silica gel. The ability of TLC to provide both the required elimination of extraneous compounds[1,5] and quantitative preliminary separation of steroids complements the GLC limitations.

The results obtained enable TLC and GLC conditions to be selected that allow the complete separation of androstanediol and pregnanediol isomers in complex mixtures of biological origin.

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EXPERIMENTAL

Instruments

The gas chromatograph utilized was a Pye Unicam 104 Model 84 (Cambridge, Great Britain), equipped with a flame ionization detector. Retention times were measured with an Infotronics CRS 104 electronic integrator (Boulder, Colo., U.S.A.), coupled with a Philips PM 8 100 1-mV full-scale deflection recorder at a speed of 10 mm/min.

Gas–liquid chromatography

The support material was Gas-Chrom Q, 80–100 mesh, supplied by Applied Science Labs. (State College, Pa., U.S.A.). Coating of the support was carried out by careful evaporation of the solvent from a slurry of the support in a solution containing the liquid phase. Silanized glass columns of 4 mm I.D. were packed under vacuum with the sieved coated support by gently tapping them with a pencil. After conditioning for 24 h at a temperature 20° higher than the operating temperature, the columns were ready for use. The operating conditions are given in Table I.

TABLE I

GAS–LIQUID CHROMATOGRAPHY CONDITIONS

The efficiency was measured by injecting 200 ng of 5α-cholestane.

Stationary phase	*Liquid phase loading (%)*	*Column length (m)*	*Number of theoretical plates*	*Carrier gas flow-rate at 2.1 bar (ml/min)*	*Temperature (°C)*
SE-30	2	1.50	3000	45	251*
OV-1	2	1.80	3000	50	208**
OV-17	2	1.50	2500	45	220***
QF-1	2	2.10	2800	50	208§
XE-60	2	1.50	2000	45	181§§
NGS	2	2.10	3500	50	215§§§
Hi-Eff 8 BP	2	1.50	2500	45	201†

* Except for CMDMSi: 281°.
** Except for CMDMSi: 231°.
*** Except for CMDMSi, Acet. and free: 256°.
§ Except for CMDMSi: 231°.
§§ Except for CMDMSi, Acet. and free: 210°.
§§§ Except for free and Acet.: 267°.
† Except for free and Acet.: 279°.

Thin-layer chromatography

Pre-coated silica gel F_{254} thin-layer plates, supplied by Merck (Darmstadt, G.F.R.), were utilized. After migration, the compounds were located by spraying with 3:7 methanol–conc. sulphuric acid and heating at 110° for 5 min. Details of the technique have been given previously[1].

Paper chromatography

Whatman No. 1 paper was washed with purified methanol and then with anhydrous benzene for 24 h and dried at 60°. Spotting was carried out under a stream

of cold air on paper that had previously been dampened with 1:1 propanediol–methanol. After migration in 1:1 cyclohexane–benzene at 23 ± 1°, the dihydroxysteroids were oxidized with chromic anhydride and then located with Zimmermann's reagent[6].

Steroids

Some steroids were synthesized by the Meerwein–Pondorf reduction[7]: 5α-androstane-3α,17α-diol by reduction of 3α-hydroxy-5α-androstan-17-one (20% yield); 5β-androstane-3α,17α-diol by reduction of 3α-hydroxy-5β-androstan-17-one (22% yield); and 5β-androstane-3β,17α-diol by reduction of 3β-hydroxy-5β-androstan-17-one (15% yield). Androst-4-ene-3β,17β- and -3α,17β-diols were obtained by reduction of 17β-hydroxyandrost-4-ene-3-one with potassium borohydride. The principal product of reduction was the 3β-isomer; the 3α-isomer was purified by TLC on silica gel F_{254} with 95:5 methylene chloride–methanol. Androst-4-ene-3β,17α- and -3α,17α-diols were prepared by the same procedure from 17α-hydroxyandrost-4-ene-3-one.

All the synthesized products were purified by chromatography on an alumina column with an elution gradient[8], using a donor mixture of 6% ethanol in benzene. Purities, after crystallization, were checked by GLC and TLC and identities were confirmed by IR spectrometry. Table II shows the origins of the C_{19} and C_{21} dihydroxysteroids.

TABLE II

ORIGIN OF DIHYDROXYSTEROIDS

Suppliers: Sigma (St. Louis, Mo., U.S.A.); Merck (Darmstadt, G.F.R.); Ikapharm (Ramat-Gan, Israel); Roussel UCLAF (Romainville, France).

No.	*Name*	*Origin*
1	5α-Androstane-3α,17α-diol	Synthesis
2	-3α,17β-diol	Sigma
3	-3β,17β-diol	Merck
4	-3β,17α-diol	Synthesis
5	5β-Androstane-3α,17α-diol	Synthesis
6	-3α,17β-diol	Merck
7	-3β,17β-diol	Ikapharm
8	-3β,17α-diol	Synthesis
9	Androst-5-ene-3β,17β-diol	Merck
10	-3β,17α-diol	Sigma
11	Androst-4-ene-3β,17β-diol	Synthesis
12	-3β,17α-diol	Synthesis
13	-3α,17β-diol	Synthesis
14	-3α,17α-diol	Synthesis
15	5α-Pregnane-3α,20α-diol	Merck
16	-3α,20β-diol	Sigma
17	-3β,20β-diol	Sigma
18	-3β,20α-diol	Sigma
19	5β-Pregnane-3α,20α-diol	Roussel UCLAF
20	-3α,20β-diol	Sigma
21	-3β,20β-diol	Sigma
22	-3β,20α-diol	Sigma
23	Pregn-5-ene-3β,20β-diol	Merck
24	-3β,20α-diol	Sigma
25	Pregn-4-ene-3β,20β-diol	Sigma

TABLE III

SEPARATION OF ANDROSTANEDIOL AND PREGNANEDIOL ISOMERS BY PAPER AND THIN-LAYER CHROMATOGRAPHY

Results are expressed as R_F values relative (R_t) to 5α-androstane-3β,17β-diol (R_t = 1.00). Solvent systems used for migrations: A = methylene dichloride–ethyl acetate (6:4); B = diethyl ether–ethyl acetate (95:5); C = benzene–ethyl acetate (1:1); D = cyclohexane–ethyl acetate (3:7); E = chloroform–diethyl ether (7:3); F = benzene–ethanol (9:1).

Steroid No.	*TLC*						*PC*
	A	*B*	*C*	*D*	*E*	*F*	
1	0.56	0.85	0.71	0.83	0.57	0.87	2.10
2	1.00	1.10	1.00	1.00	0.95	1.15	1.65
3	1.00	1.00	1.00	1.00	1.00	1.00	1.00
4	0.88	0.99	0.91	0.97	0.89	0.95	1.26
5	0.28	0.49	0.43	0.55	0.31	0.59	1.40
6	0.63	0.62	0.61	0.72	0.63	0.77	1.08
7	1.18	1.19	1.10	1.12	1.07	1.12	1.40
8	0.85	1.04	0.88	0.97	0.84	0.95	1.87
9	1.03	1.08	1.03	1.04	1.03	0.95	0.90
10	0.96	1.07	1.00	1.04	0.97	0.92	0.75
11	1.12	1.13	1.09	1.08	1.09	0.99	1.20
12	1.00	1.08	1.00	1.08	0.95	0.95	1.44
13	0.73	0.86	0.65	0.72	0.75	0.83	1.00
14	0.39	0.64	0.48	0.62	0.38	0.69	1.10
15	0.90	1.04	0.95	1.02	1.00	1.02	
16	0.98	1.00	0.97	1.02	1.05	1.06	
17	1.06	1.05	1.00	1.08	1.05	1.10	
18	0.98	0.93	0.98	1.00	1.05	1.11	
19	0.58	0.63	0.67	0.69	0.66	0.90	1.65
20	0.72	0.84	0.81	0.84	0.79	0.97	
21	1.16	1.24	1.16	1.22	1.12	1.38	
22	1.12	1.21	1.06	1.14	1.11	1.33	
23	1.15	1.14	1.10	1.12	1.08	1.29	
24	1.04	1.05	1.10	1.11	1.06	1.32	
25	1.10	1.14	1.04	1.10	1.08	1.36	

TABLE IV

SEPARATION FACTORS FOR ANDROSTANEDIOL AND PREGNANEDIOL ISOMERS ON THIN-LAYER AND PAPER CHROMATOGRAMS

The separation factor is the ratio of the relative R_F value (R_t) of compound a to that of compound b. 5α-A- = 5α-androstane; 5α-Pr- = 5α-pregnane.

Steroid		*TLC*						*PC*
a	*b*	*A*	*B*	*C*	*D*	*E*	*F*	
5α-A-3α,17β	5β-A-3β,17β	0.85	0.92	0.91	0.89	0.89	1.03	1.18
5α-A-3α,17β	5β-A-3α,17β	1.58	2.20	1.64	1.43	1.50	1.49	1.52
5α-A-3α,17β	5α-A-3β,17β	1.00	1.10	1.00	1.03	0.95	1.15	1.65
5β-A-3α,17β	5β-A-3α,17α	2.25	1.47	1.24	1.31	2.03	1.30	—
5α-Pr-3α,20α	5β-Pr-3α,20α	1.55	1.65	1.42	1.50	1.48	1.14	
5α-Pr-3α,20α	5α-Pr-3β,20α	0.91	1.07	0.97	1.02	0.95	0.92	

Derivative formation

Acetates (Acet.). The steroid was allowed to react with a 1:1 acetyl chloride–pyridine mixture for 12 h.

Chloromethyldimethylsilyl ethers (CMDMSi). These derivatives were prepared according to Thomas and co-workers[9,10].

Trimethylsilyl ethers (TMSi). The silylation was carried out with 200 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 20 μl of distilled pyridine for 1 h at 60°[11,12].

Trifluoroacetates (TFA). The steroid was allowed to react with 200 μl of 1:10 trifluoroacetic anhydride–tetrahydrofuran for 1 h at 60°.

Heptafluorobutyrates (HFB). The reagent was 1 ml of 1:1:10 heptafluorobutyric anhydride–tetrahydrofuran–*n*-hexane. The mixture was left for 1 h at 60° in the presence of the dry steroid.

The derivatives were prepared using 50 μg of each steroid. After the reaction, the mixture was evaporated to dryness under nitrogen at 60°. The dry extract was dissolved in 200 μl of carbon disulphide and 2 μl of this solution were injected on the GLC column.

Retention time data

The corrected retention times were measured with an electronic integrator with an accuracy of ± 1 sec. The steroid number was calculated using 5α-cholestane (relative retention time = 1.00; *SN* = 27.00), 5α-androstane (*SN* = 19.00) and 5α-pregnane (*SN* = 21.00) as standards for the measurements. Steroid numbers were rounded off to the nearest 5 units.

The *SN*, being logarithmic, is additive and therefore a change in *SN*, expressed as ΔSN, reflects chemical alterations to a functional group. The symbols ΔSN_g and ΔSN_r are used to denote addition and substitution of a functional group, respectively.

RESULTS

Thin-layer and paper chromatography

The results are presented in Table III. TLC permits the C_{19} and C_{21} dihydroxysteroids to be separated easily into 5α- and 5β-zones. The sequence of decreasing polarity is: 3β,5β- $<$ 3β,Δ^5- $\approx$ 3β,5α- $<$ 3α,5α- $<$ 3α,5β-. These results are in good agreement with those published elsewhere[13]. It is noted that the 17β- and 20β-hydroxy isomers have a higher mobility than the corresponding 17α- and 20α-hydroxy compounds.

Table IV gives separation factors for some isomeric pairs and it is clear that if the separation of 5α- and 5β-isomers is easy, the resolution of 3α- and 3β-androstanediols in the 5α-androstane series is difficult. The separations obtained on pre-coated silica gel with solvent systems B and F are different from those obtained on silica gel G[14,15]. We used pre-coated silica gel F_{254} because the results obtained were very reproducible. The 3α- and 3β-androstanediols can be resolved by paper chromatography (separation factor = 1.65) in 1:1 cyclohexane–benzene or by TLC on alumina G in 97:2.85:0.15 benzene–ethanol–water[16]. In any system, 5α-androstane-3β,17β-diol and androst-5-ene-3β,17β- diol were not separated. Consequently, complementary analytical methods were necessary in order to obtain a complete resolution of C_{19} and C_{21} dihydroxysteroids.

TABLE V
STEROID NUMBERS OF ANDROSTANEDIOL AND PREGNANEDIOL ISOMERS BY GLC

Stationary phase	*Derivative*	*5α-Androstanediol-*				*5β-Androstanediol-*			
		3α-		*3β-*		*3α-*		*3β-*	
		*17α- (1)**	*17β- (2)*	*17β- (3)*	*17α- (4)*	*17α- (5)*	*17β- (6)*	*17β- (7)*	*17α- (8)*
SE-30	Free	23.75	23.80	24.00	23.80	23.50	23.50	23.50	23.50
	Acetate	25.40	25.70	26.40	26.10	25.10	25.65	25.75	24.70
	TMSi	23.10	24.15	24.80	24.45	22.85	24.10	24.10	23.10
	TFA	20.50	21.45	22.20	21.65	20.30	21.80	21.40	20.45
	HFB	20.40	21.90	23.15	22.15	20.55	22.20	22.30	20.60
	CMDMSi	28.60	30.10	31.10	30.30	28.45	30.10	30.20	29.00
OV-1	Free	23.95	24.00	24.15	24.05	23.75	23.80	23.50	23.50
	Acetate	25.90	26.20	26.70	26.40	25.50	26.15	26.00	25.60
	TMSi	23.70	24.60	24.60	24.05	23.55	24.70	24.45	23.60
	TFA	21.20	21.90	22.70	22.15	21.00	22.05	21.90	21.00
	HFB	21.30	22.70	23.40	22.90	21.40	23.00	23.00	21.50
	CMDMSi	29.05	30.20	31.50	30.55	28.75	30.15	30.20	29.05
OV-17	Free	26.30	26.40	26.45	26.45	25.90	25.95	26.00	25.85
	Acetate	27.70	28.00	28.70	28.40	27.40	27.80	27.80	27.50
	TMSi	23.30	23.90	24.90	24.45	23.20	24.05	23.90	23.25
	TFA	21.45	22.00	22.75	22.45	21.65	22.05	21.95	21.40
	HFB	20.30	21.30	21.95	21.55	20.35	21.30	21.35	20.20
	CMDMSi	29.90	31.00	32.30	31.60	29.70	31.00	31.00	30.00
QF-1	Free	27.65	27.75	28.10	28.00	27.65	27.95	27.40	27.25
	Acetate	31.00	31.60	32.10	31.80	30.65	31.45	32.10	31.10
	TMSi	23.00	23.90	25.20	24.40	22.80	24.00	23.90	22.80
	TFA	26.80	27.30	28.35	27.60	26.80	27.50	27.35	26.50
	HFB	26.70	27.80	29.40	28.20	26.80	28.30	28.30	26.90
	CMDMSi	31.50	32.70	34.10	33.20	31.20	32.75	32.80	31.60
XE-60	Free	30.00	30.20	30.70	30.30	30.00	30.20	29.90	29.55
	Acetate	30.45	30.85	31.40	31.00	30.00	30.85	30.80	30.10
	TMSi	23.15	24.00	25.20	24.60	23.15	24.45	23.85	22.85
	TFA	24.90	25.40	26.20	25.60	24.80	25.75	25.45	24.60
	HFB	23.40	24.50	26.00	24.75	23.45	24.95	25.05	23.25
	CMDMSi	31.95	33.20	34.65	33.80	31.70	33.55	33.25	31.90
NGS	Free	33.00	33.30	33.90	33.60	32.90	33.20	32.80	32.50
	Acetate	31.80	31.90	32.90	32.40	31.70	32.20	31.80	31.10
	TMSi	21.95	22.60	24.15	23.40	22.30	23.55	21.95	21.35
	TFA	24.00	24.50	25.65	25.05	24.20	25.10	24.50	23.45
	HFB	21.50	22.25	23.85	22.70	21.55	23.00	22.80	20.90
Hi-Eff 8 BP	Free	32.80	32.95	33.35	33.20	32.50	32.75	32.40	32.20
	Acetate	30.05	31.00	32.00	31.60	30.70	31.20	30.80	30.10
	TMSi	21.80	22.40	24.05	23.15	21.95	23.45	22.40	21.40
	TFA	22.25	22.65	23.80	23.40	22.40	23.40	22.80	21.65
	HFB	19.75	20.60	22.25	20.95	20.05	21.30	21.10	19.20

* Numbers in parentheses refer to identification numbers in Table II.

Androst-5-ene-diol-3β-		*5α-Pregnanediol-*				*5β-Pregnanediol-*			
		3α-		*3β-*		*3α-*		*3β-*	
17β- (9)	*17α- (10)*	*20α- (15)*	*20β- (16)*	*20β- (17)*	*20α- (18)*	*20α- (19)*	*20β- (20)*	*20β- (21)*	*20α- (22)*
23.35	23.35	25.85	25.65	25.55	25.90	25.45	25.25	25.40	25.40
26.25	26.10	27.85	27.70	28.10	28.20	27.75	27.45	27.45	27.95
25.00	24.45	26.35	26.10	27.00	27.15	26.55	26.25	26.25	27.05
21.95	21.45	23.40	23.05	23.80	24.00	23.45	22.60	22.60	23.75
23.05	21.95	24.00	23.25	24.80	25.15	24.15	23.35	23.55	24.80
31.05	30.40	32.45	32.10	32.70	33.40	32.40	31.60	32.60	33.20
23.85	23.85	25.85	25.70	25.75	25.95	25.80	25.55	25.20	25.65
26.80	26.20	28.30	28.00	28.70	28.90	28.30	28.00	27.70	28.55
25.00	24.95	26.85	26.60	27.50	27.60	27.00	26.75	26.60	27.35
22.60	22.05	23.95	23.65	24.25	24.65	23.95	23.65	23.65	24.30
23.85	23.65	24.85	24.20	25.45	25.95	25.05	24.35	24.40	25.50
31.40	30.55	32.80	32.40	33.30	33.60	32.80	32.40	32.40	33.30
26.60	26.80	28.50	28.10	28.30	28.30	27.90	27.70	27.70	27.90
28.80	28.50	30.20	30.00	30.50	30.70	30.00	29.80	29.80	29.95
24.95	24.50	26.15	25.95	26.90	27.00	26.30	26.00	25.90	26.05
22.55	22.25	24.00	23.80	24.50	24.70	23.75	23.50	23.20	23.60
21.65	21.40	23.00	22.55	23.80	24.05	23.00	22.50	22.55	23.80
32.20	31.60	33.70	33.35	34.70	34.80	33.60	33.30	33.30	33.30
27.80	27.80	30.05	29.45	29.60	30.10	30.10	29.40	28.75	29.50
31.65	31.65	33.60	33.20	33.85	34.05	33.30	32.70	33.10	33.90
24.80	24.10	26.50	26.10	27.30	27.60	26.70	26.20	26.50	27.30
27.75	27.15	29.30	28.90	29.80	30.40	29.65	29.00	28.90	29.80
28.80	27.80	29.80	29.00	30.55	31.30	30.10	29.25	28.95	29.40
33.70	32.80	35.20	34.80	36.10	36.40	35.40	34.80	34.80	36.10
30.75	30.50	32.20	31.55	32.10	32.50	32.20	31.85	31.50	31.95
31.30	31.05	33.00	32.80	33.15	33.40	32.90	32.50	32.40	33.20
25.15	24.60	26.40	26.20	27.30	27.65	27.00	26.80	26.00	26.15
25.90	25.45	27.45	27.05	27.70	28.20	27.70	27.25	27.05	27.15
25.60	24.55	26.55	25.90	27.20	27.75	26.85	26.15	26.15	27.10
34.55	33.70	36.00	35.60	37.10	37.30	36.40	35.90	35.55	36.90
34.40	34.10	35.00	34.30	34.90	35.50	35.00	34.30	34.20	34.80
32.75	32.60	34.00	33.70	34.80	35.00	34.30	33.80	33.60	33.90
24.25	23.55	25.30	24.95	26.55	26.95	26.20	25.85	24.90	26.00
25.40	24.90	26.70	26.30	27.30	27.80	27.15	26.70	26.40	27.15
23.70	22.60	24.55	24.00	25.35	26.00	25.05	24.45	24.00	24.50
33.60	33.55	34.95	34.40	34.85	35.40	34.70	34.20	33.65	34.40
31.90	31.60	33.30	32.90	34.00	34.25	33.50	33.30	32.90	32.90
24.10	23.40	24.95	24.60	26.25	26.80	25.95	25.60	24.65	25.10
23.60	23.05	24.80	24.40	25.65	26.05	25.50	25.00	24.45	24.90
22.00	20.85	22.85	22.20	23.65	24.25	23.45	22.85	22.35	23.10

Gas–liquid chromatography

The *SN*s of isomeric androstanediols and pregnanediols obtained by GLC are given in Table V. We used this expression of retention data rather than the Kováts[17] retention index (*RI*) because the increments of *SN* with temperature are smaller than the *RI* increments[18]. The use of the *SN* enables results obtained in different laboratories to be compared without excessive errors. Nevertheless, the conversion of *SN* into *RI* is always possible, after the determination of the relationship between *RI* and *SN* on the liquid phase used[19].

The relative polarities of liquid phases, measured by the difference in retention times between an *n*-alkane (*n*-octacosane) and 5α-cholestane, can be expressed as follows: SE-30 ≈ OV-1 < OV-17 < QF-1 < XE-60 < NGS < Hi-Eff 8 BP.

The CMDMSi derivatives, which have a high polarity, are considerably retained on a polar column; for these derivatives, the response of electron capture detection is low. Therefore, their utilization in GLC–mass spectrometry with multiple ion detection is of great interest, their relative isotopic abundance (^{35}Cl:^{37}Cl) being utilized as a guide to the possible incidence of interfering background[20–22].

The free steroids and the acetate derivatives are difficult to chromatograph through a polar liquid phase and consequently were not utilized for GLC analytical work.

The most useful derivatives for GLC work with hydroxy-substitued steroids are the TMSi ethers. The hydroxyl groups of the steroids investigated are not sterically hindered and are easily converted into the TMSi ethers. These ethers have excellent properties for GLC separation; the bulky TMSi group provides steric shielding of the oxygen atom. With non-selective phases, the TMSi ethers were eluted after the parent steroids, but the order was reversed with selective phases. These derivatives allowed a good resolution of C_{19} and C_{21} dihydroxysteroids on polyester liquid phases.

The TFA derivatives were also suitable for routine work because of their rapid preparation and short retention times. These derivatives were often eluted before the TMSi ethers[23,24], except on QF-1, XE-60 and NGS phases. The resolution between the isomers was not as good as that obtained with the TMSi derivatives.

The HFB derivatives, introduced in 1963 by Clark and Wotiz[25], are suitable for GLC with electron capture detection. These derivatives have non-polar properties on the polyester phases NGS, Hi-Eff 8 BP and XE-60. On QF-1, the HFB were eluted after the parent steroids, this phase being specific for keto and ester groups[26].

The interaction between a functional group and the liquid phase was expressed by the function $\Delta SN_g^{(\text{polar phase})} - \Delta SN_g^{(\text{OV-1})}$ or $\Delta SN_r^{(\text{polar phase})} - \Delta SN_r^{(\text{OV-1})}$ (ref. 27).

The $\Delta SN_g^{(\text{polar phase})} - \Delta SN_g^{(\text{OV-1})}$ values obtained by the addition of two hydrogen atoms to the androst-5-ene nucleus are presented in Fig. 1. It is clear that the elution order for an androst-5-ene steroid and the corresponding saturated (A/B *trans*) compound is dependent upon both the phase and the derivative. Non-selective phases showed very little ability to distinguish between such compounds, whereas the polyester phases retained the unsaturated compound somewhat better than the saturated compound. The reverse was true with QF-1, which allowed a good separation of compounds Nos. 3 and 9 when HFB and TMSi derivatives were used.

The $\Delta SN_r^{(\text{polar phase})} - \Delta SN_r^{(\text{OV-1})}$ values obtained by the substitution of the 5β- by the 5α-androstane isomer are presented in Fig. 2. The interaction between the polyester phases and the TMSi functions allowed a good resolution of 5α- and 5β-

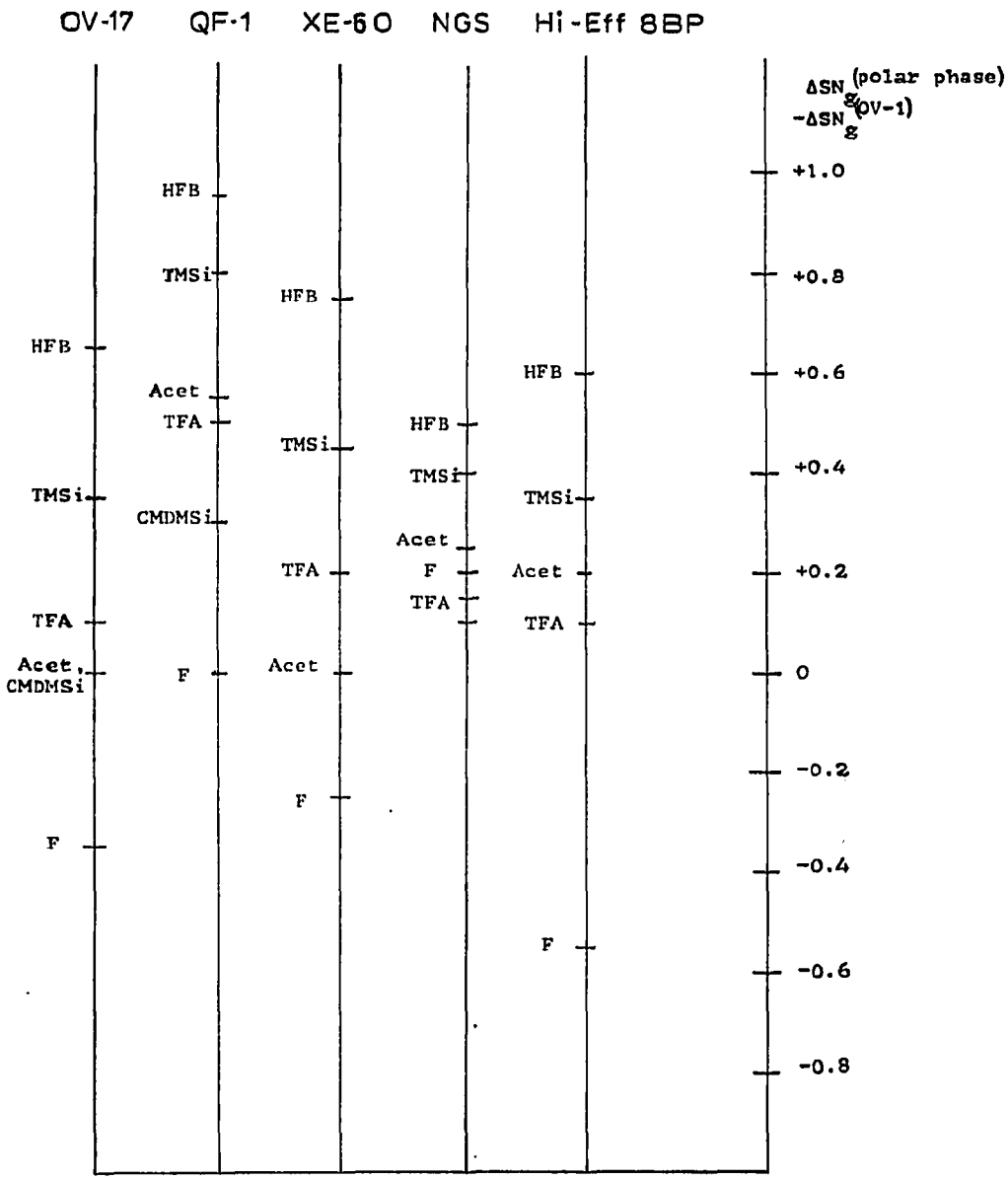

Fig. 1. Distribution of retention data obtained by 5α-reduction of the androst-5-ene nucleus.

isomers. The separation of these isomers was virtually independent of the nature of the nucleus (Table V).

The GLC behaviour of pregnanediol isomers has been reviewed by Vandermolen[28]. There was not complete agreement between the results he gave and our results. Moreover, the sequence of elution for the various isomers chromatographed under apparently similar conditions differed. Table VI presents some of these results.

It was clear that the identification of a pregnanediol isomer might be incorrect

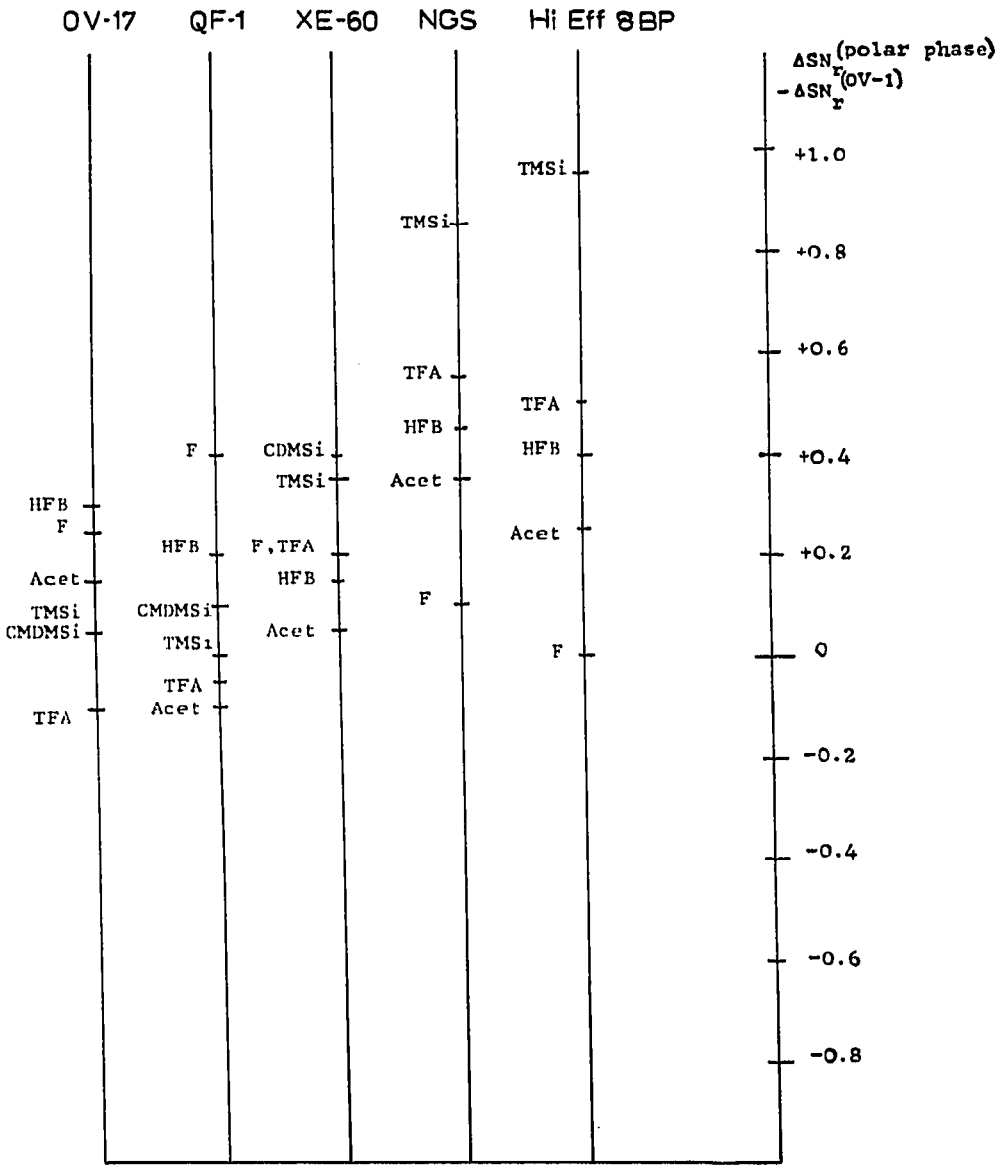

Fig. 2. Distribution of retention data obtained by substitution of the 5β- to the 5α-isomer.

if retention data from the literature are used. In the 5α-series, the 3α-hydroxy compounds (axial conformation) had higher mobilities than the corresponding 3β-hydroxy compounds (equatorial conformation). The 20α-hydroxy isomer (*S*-configuration) had a higher polarity than the 20β-hydroxy isomer. Consequently, on all polar phases, the sequence of elution of TMSi, TFA and HFB derivatives in the 5α-pregnane series was: 3α,20β-; 3α,20α-; 3β,20β-; 3β,20α-. In the 5β-pregnane series, the pattern observed for the 3-hydroxy compounds was reversed. On polar phases, the order of

TABLE VI

COMPARISON OF SEQUENCE OF ELUTION OF PREGNANEDIOL ISOMERS

The pregnanediols are identified as in Table II.

Stationary phase	*Derivative*	*Sequence of elution*	*Operating conditions*		
			Column length (ft)	*Phase loading (%)*	*Temperature (°C)*
QF-1	Free	20 17 19 18	4.5	1	200
		21 20 16 22 17 15 19 18	3	6	250
		21 16 20 22 17 15 19 18	6	1	210
		20 17 19 15 18	6	1	175
		21 20 16 22 17 15 19 18*			
	TMSi	20 15 19 17 18	6	1	175
		20 16 15 19 17 18	3	6	250
		16 20 21 15 19 22 17 18*			
	Acet	20 21 16 19 17 16 15 18	6	3	210
		20 21 19 16 22 15 17 18	3	6	250
		20 21 16 19 15 17 22 18*			
SE-30	Free	21 20 22 19 17 16 15 18	6	3	210
		20 19 17 15 18	6	1	224
		20 21 22 19 17 16 15 18*			
	Acet	21 20 16 22 15 19 17 18	6	3	220
		20 19 15 17 18	6	1	224
		21 20 16 19 15 22 17 18*			
XE-60	Free	21 16 20 22 17 19 15 18	5	3	245
		20 19 17 15 18	6	1	213
		20 15 17 19 18	6	1	215
		21 16 20 22 17 19 15 18*			
	Acet	21 20 22 16 19 15 17 18	6	1	215
		20 19 15 17 18	6	1	218
		21 20 16 19 15 17 22 18*			
	TMSi	15 20 19 17 18	6	1	218
		20 15 19 17 18	6	1	205
		15 20 19 17 18	6	1	202
		21 22 16 15 20 19 17 18*			
NGS	TMSi	15 19 20 17 18	6	1	207
		21 16 15 20 22 19 17 18*			
Hi-Eff 8 BP	TFA	15 22 19 18	9	2.75	230
		16 21 15 22 20 19 17 18*			
	TMSi	15 22 19 18	9	2.75	230
		16 21 15 20 22 19 17 18*			

* This work. All other results are taken from ref. 28.

elution was generally the following: 3β,20β-; 3β,20α- $\approx$ 3α,20β-; 3α,20α-.

These differences in the sequence of elution for the pregnane-3ξ,20ξ-diol isomers were probably due to the influence of the analysis temperature. Work in progress has shown that the retention times of 5β-pregnane-3β,20α-di-TMSi relative to those of 5α-pregnane-3α,20α-di-TMSi on a Dexsil column were 0.994, 0.990 and 0.971 at 289°, 280° and 214°, respectively. These results suggested that the $\delta SN/\delta T$ relationships for these two compounds were different. The continuation of this work will be published elsewhere.

DISCUSSION

This study of TLC and GLC behaviour shows that GLC is unable to separate easily all of the different urinary C_{19} and C_{21} dihydroxysteroids.

Fig. 3 shows the *SN*s of the TMSi derivatives of androstanediol and pregnanediol isomers on Hi-Eff 8 BP, which is the most specific liquid phase for this analysis. This table shows that many pairs of steroids are not resolved under the conditions used : 5α-androstane-3α,17β-diol and 5β-androstane-3β,17β-diol; 5β-androstane-3α,17β-diol and androst-5-ene-3β,17α-diol; 5α-androstane-3β,17β-diol and androst-5-ene-3β,17β-diol; and 5β-pregnane-3β,20β-diol and 5α-pregnane-3α,20β-diol.

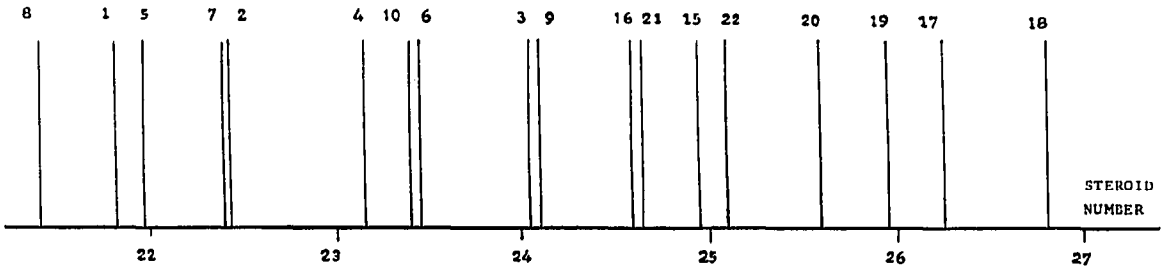

Fig. 3. Schematic representation of retention data of the TMSi derivatives of androstanediol and pregnanediol isomers on Hi-Eff 8 BP. The steroids are numbered as in Table II. Conditions permitted two compounds to be separated with 98% resolution when the ΔSN was 0.25.

The combination of TLC and GLC permitted the resolution of two steroid pairs.

Fig. 4 shows a chromatogram of dihydroxysteroids extracted from human urine; the urinary extract was not purified by TLC. It is clear that the quantification of 5β-androstane-3α,17β-diol was impossible, as the conditions used failed to separate this compound from androst-5-ene-3β,17α-diol. Consequently, the urinary extract was separated by TLC on silica gel F_{254} in system A into two zones, the "5α-zone" ($0.80 \leqslant R_f \leqslant 1.15$) and the "5$\beta$-zone" ($0.50 \leqslant R_f \leqslant 0.80$). After this separation, the chromatograms shown in Fig. 5 were obtained. By this method, we showed that 5β-androstane-3α,17β-diol measured without a preliminary purification by TLC was contaminated with approximately 12% of androst-5-ene-3β,17α-diol (3–22%)[29].

5β-Androstane-3β,17β-diol, which interferes with 5α-androstane-3α,17β-diol in GLC, is partially eliminated from the "5α-zone" by TLC (Table III). However, we think that this interference may be neglected because the 3β–5β structure is very improbable in urines.

The combination of GLC and TLC (Fig. 5) permits six dihydroxysteroids to be specifically measured: 5α-androstane-3α,17β-diol, androst-5-ene-3β,17α- and -3β,17β-diol, 5α-pregnane-3α,20α-diol (peaks 2, 10, 9 and 15 of the "5α-zone"), 5β-androstane-3α,17β-diol and 5β-pregnane-3α,20α-diol (peaks 6 and 19 of the "5β-zone"). The homogeneity of peaks 2, 10, 15, 6 and 19 was confirmed by utilizing different stationary phases and derivatives[1]. A supplementary proof of the identity of these steroids was obtained by coupling a high-resolution glass capillary column with a mass spectrometer[30].

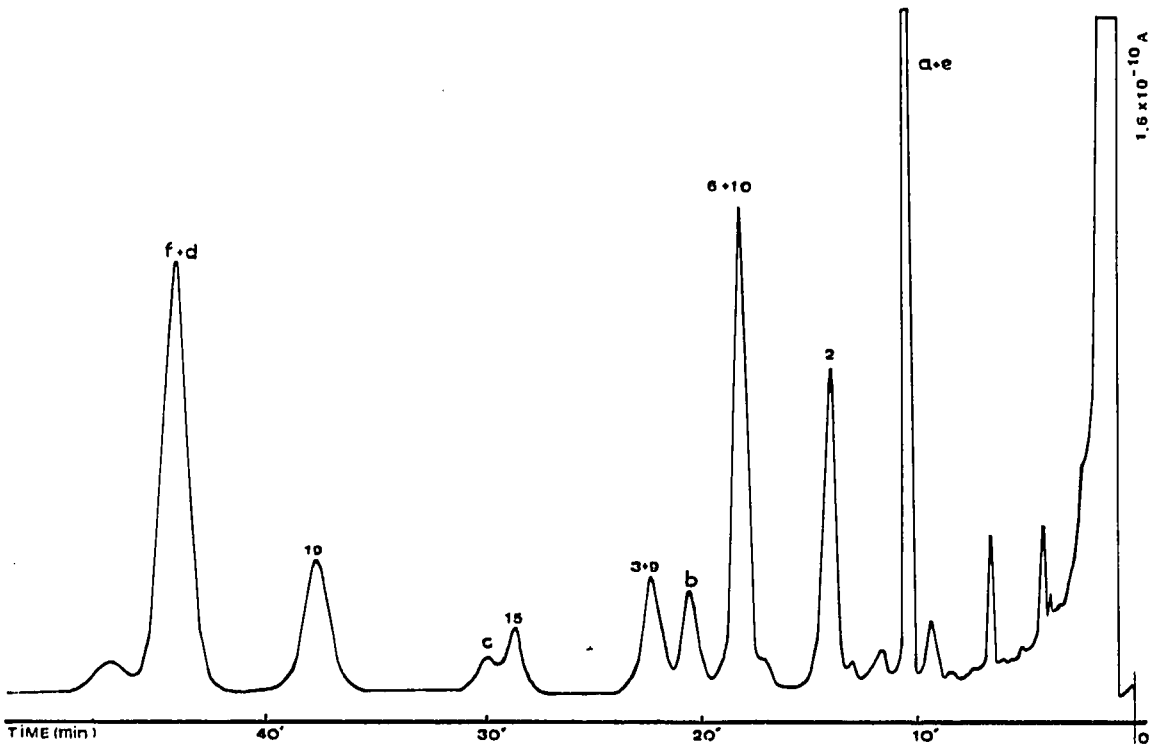

Fig. 4. Chromatogram of TMSi derivative from a male urinary extract obtained after purification according to the method described in Fig. 7, but without preliminary separation on silica gel F_{254}. This chromatogram was obtained on a 2.70 m × 4 mm glass column coated with 1.6% Hi-Eff 8 BP on Gas-Chrom Q, 100–120 mesh. Temperatures: oven, 207°; detector, 250°. Gas flow-rate: 40 ml/min. The peaks are numbered as in Table II. Peaks a, b, c, d, e and f were not identified.

Fig. 5 shows that the quantitation of 5α-androstane-3β,17β-diol was impossible, this steroid not being separated from androst-5-ene-3β,17β-diol. In order to identify this testosterone metabolite, the physiological importance of which was recently stressed[31,32], we have developed a method[2] that allows an unequivocal separation between this compound and androst-5-ene-3β,17β-diol (Fig. 6). The unsaturated compounds were eliminated by epoxidation followed by paper chromatography. The procedure can be summarized as follows (see Fig. 7): trace amounts of [4-^{14}C]-5α-androstane-3β,17β-diol and [1,2-^{3}H]-5α-androstane-3α,17β-diol were added to a one-fifth aliquot of 24-h urines for recovery determinations. The mixture was incubated with *Helix pomatia* β-glucuronidase at 37° and the free steroids were extracted with diethyl ether. The use of Girard's T reagent permitted all ketosteroids to be eliminated, the hydroxysteroids alone being purified by adsorption chromatography on alumina. The purified hydroxysteroid mixture was divided into halves. One half was submitted to TLC, in which two zones were obtained that were analyzed by GLC on Hi-Eff 8 BP after trimethylsilylation. Epoxidation with 3-chloroperbenzoic acid was carried out on the second half and the reaction product was chromatographed on paper. The zone corresponding to 5α-androstane-3β,17β-diol was eluted and purified by TLC and the final extract was submitted to trimethylsilylation and analyzed by GLC on Hi-Eff 8

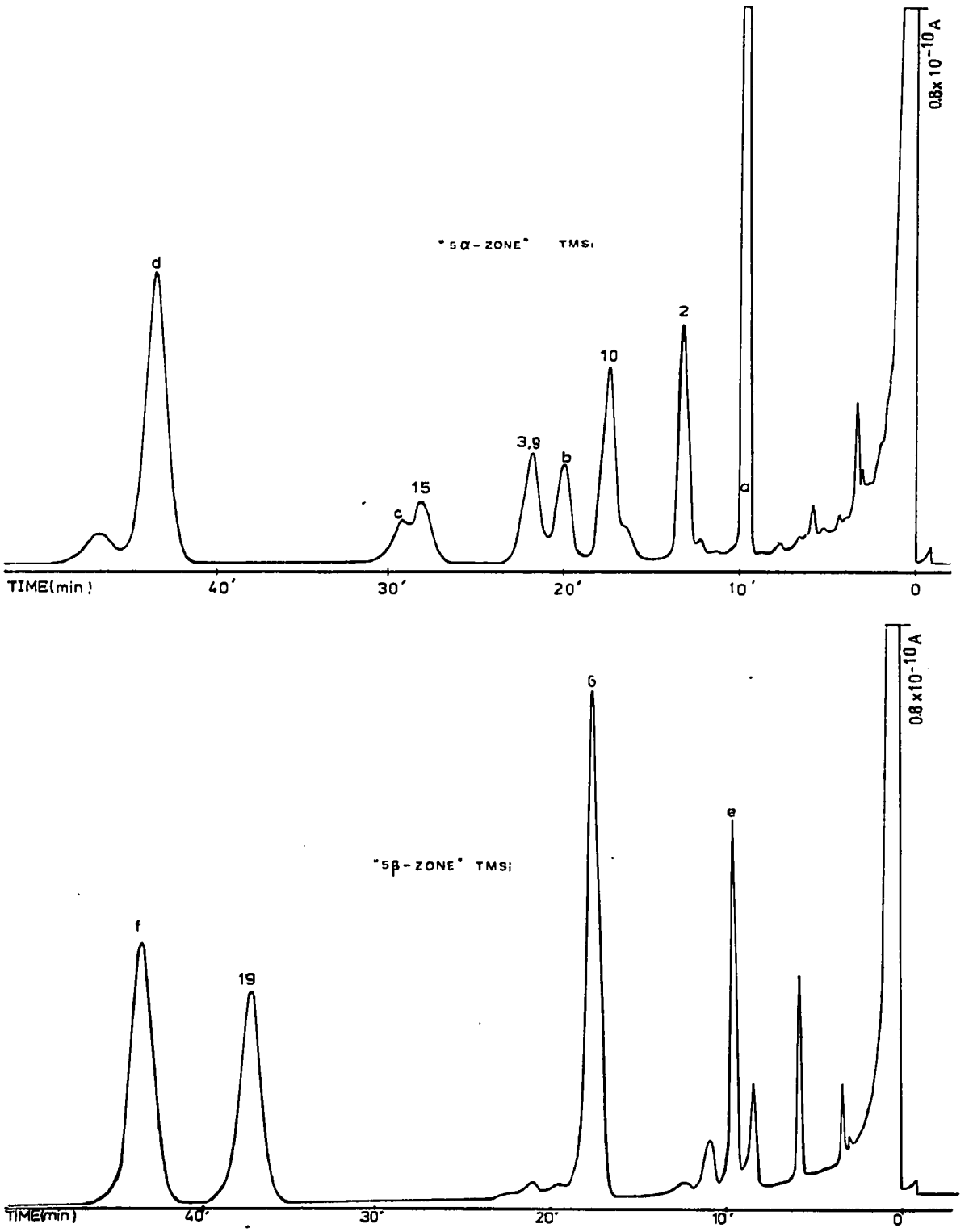

Fig. 5. Gas chromatogram of the same urinary extract as in Fig. 4, but after separation by TLC into two zones, the "5α-zone" (above) and the "5β-zone" (below). Gas chromatographic conditions as in Fig. 4.

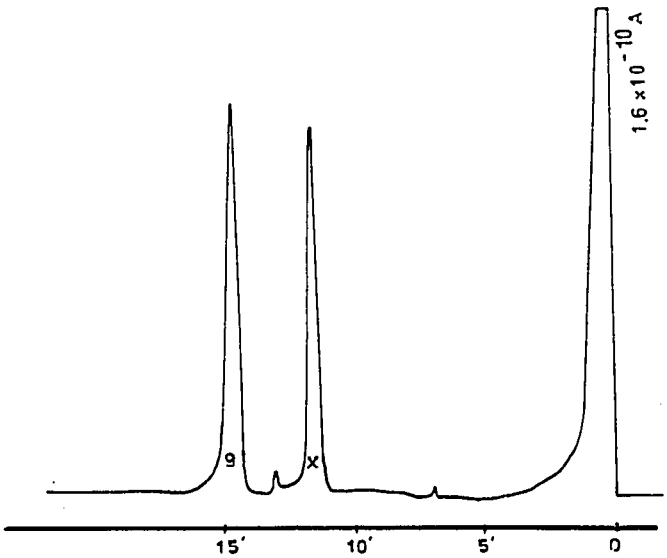

Fig. 6. Gas chromatogram of a male urinary extract after epoxidation and paper chromatography. Peak X was not identified. Analytical conditions as in Fig. 4.

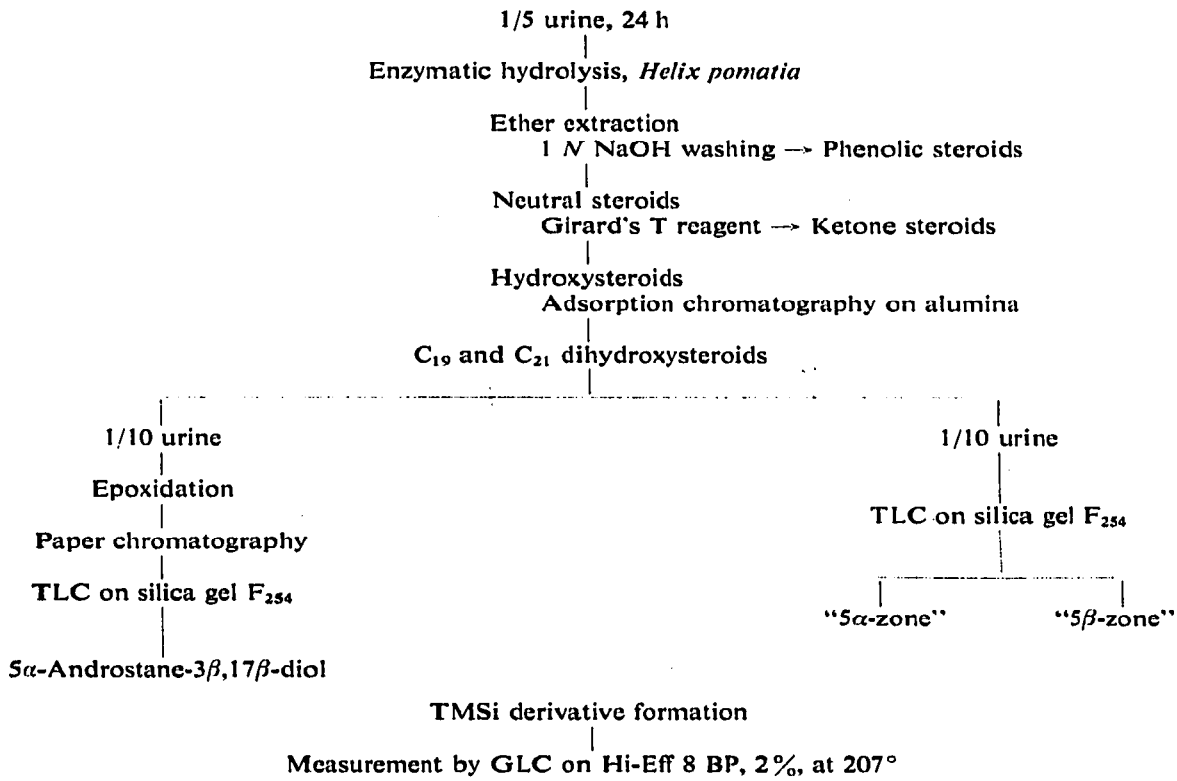

Fig. 7. Flow-scheme of the method used for the simultaneous determination of urinary androstanediols and pregnanediols.

BP. Average recoveries were 65 ± 5% and 32 ± 7% for 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol, respectively. The paper chromatographic purification step resulted in a decrease in the recovery of the latter androstanediol. Details of this method have been given elsewhere[1,2].

Although these methods were very specific[2], we have investigated another less time-consuming method. The use of QF-1 liquid phase (or Dexsil, SP-2250) with HFB, TFA or TMSi derivatives enhanced the resolution of saturated and unsaturated steroids (Fig. 8). The epoxidation reaction, previously described[2,33] for these compounds, may be not utilized.

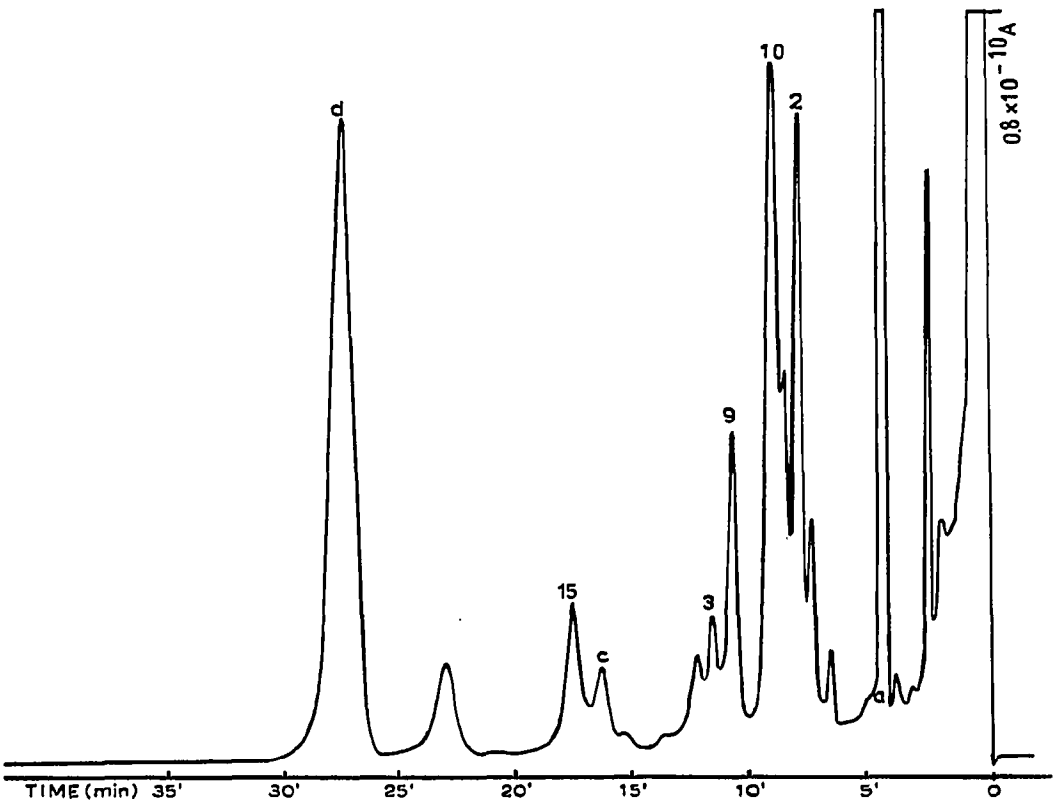

Fig. 8. Chromatogram of the "5α-zone" TMSi derivative on a QF-1 liquid phase. Peaks 3 and 9 were resolved. Glass column (2.50 m × 3 mm) coated with 1.63% QF-1. Oven temperature, 172°; injector and detector temperature, 210°; gas flow-rate, 45 ml/min.

The GLC and TLC results obtained in this study permitted specific methods to be selected that allowed many dihydroxylated urinary metabolites of testosterone (5α-androstane-3α,17β- and -3β,17β-diols)[32,34], dehydroepiandrosterone (Δ⁵-androstenediols)[35] and progesterone (5α- and 5β-pregnanediols)[36] to be analyzed.

Investigations on the separation of C_{19} and C_{21} dihydroxysteroids are in progress with the use of high-resolution glass capillary columns and new liquid phases.

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