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SEPARATION OF C_{19} AND C_{21} DIHYDROXYSTEROIDS BY OPEN-HOLE TUBULAR GLASS COLUMNS AND LIPOPHILIC GEL CHROMATOGRAPHY

F. BERTHOU, D. PICART, L. BARDOU and H. H. FLOCH
Laboratoire de Biochimie, Faculté de Médecine, B.P. 815 29279 Brest (France)
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SUMMARY

The combination of lipophilic gel chromatography and high-resolution glass capillary chromatography has been shown to be an important means of identification and quantification of steroid urinary metabolites. Some problems in the use of capillary columns, such as coating with polar and apolar stationary phases, column performance, thermal stability and injection systems, have been investigated and this type of chromatography has been used in the analysis of 33 dihydroxysteroids. Gel chromatography is a method complementary to high-efficiency gas chromatography. The steroids are eluted according to the number of hydroxyl groups in the molecules.

INTRODUCTION

At present, in almost all gas chromatographic (GC) methods used in biological studies, whether for separation alone or identification by means of gas chromatography–mass spectrometry (GC–MS), packed glass columns and glass-lined systems are employed. These columns, prepared according to Horning *et al.*[1], usually have an efficiency of 6000–7000 theoretical plates for a typical 12 ft. 1% SE-30 column[2]. However, the separation of mixtures such as urinary steroids on packed GC columns is not yet satisfactory[3], as complete separation of structurally related compounds and non-steroidal lipid contaminants is not generally achieved.

The development of thermostable open-tubular glass capillary columns suitable for the high-resolution separation of mixtures of biological origin has long been sought. In recent years, many workers[4–8] have described coating methods to be used after surface modification. The capillary columns obtained have been tested in steroid separations by Völlmin and co-workers[9–11], Novotný and Zlatkis[12,13], Ros and Sommerville[14] and German and Horning[15], especially in human urinary steroid profiles.

Until now, the application of only columns coated with apolar phases (SE-30, OV-101, OV-1) has been reported. Recently, however, the preparation of polar phase coatings has been described[16–18], which offers the best prospects for high-specificity analysis. In chromatography, the resolution is dependent on the selectivity of the

stationary phase and on the efficiency of the columns. Capillary columns enable high efficiencies to be attained and the use of polar stationary phases give high selectivity.

In this paper, the separation of 33 C_{19} and C_{21} dihydroxysteroids with high-efficiency and high-specificity glass capillary columns is described. Many problems encountered in the use of open-hole tubular columns are discussed, such as the preparation of apolar (OV-101) and polar (OV-225) columns, column performance and stability, and injection systems. The choice of derivatives such as trimethylsilyl ethers (TMS), trifluoroacetates (TFA) and heptafluorobutyrates (HFB) is discussed on the basis of the retention index.

Human urine has been shown[3] to contain many dihydroxylated steroid metabolites of testosterone. Progress in the study of the physiological significance of these metabolites is dependent on available methodology, and the development of suitable open-tubular glass capillary columns combined with liquid column chromatography on lipophilic dextran gels represents a significant advance in this field.

The aim of this work was to simplify the procedure for the identification and the quantification of urinary C_{19} and C_{21} dihydroxysteroids. This was accomplished by using lipophilic gel chromatography and glass capillary columns.

EXPERIMENTAL

Gas chromatography

Apolar column preparation. Prior to the drawing procedure, Pyrex tubes (6 × 3 mm), supplied by Sovirel (Levallois-Perret, France), were rinsed with chromic–sulphuric acid and acetone and then sealed. Glass capillaries were drawn with a Hupe and Busch machine. The capillaries were silanized by filling about 25% of the column volume with a mixture of hexamethyldisilazane (HMDS)–trimethylchlorosilane (TMCS) (5:1, v/v). After pressing this liquid plug through the column, both ends were sealed and the column was maintained at 205° for a few days. The excess of reagent was then removed by purging with nitrogen and the column was washed with toluene and methanol and dried under a stream of nitrogen.

The coating of the column was carried out according to the "static method" described by Bouche and Verzele[19]. Using *n*-hexane as solvent and a pressure of 2 mm Hg, the coating of a 25 m × 0.25 mm column (No. 1) in a water-bath at 40° was completed in 2 days. Using methylene chloride as solvent, the coating of a 40 m × 0.39 mm column (No. 3) at 22° and a 55 m × 0.37 mm (No. 4) at 18° was completed in 2.5 and 5.2 days, respectively. Some columns were treated with surface-active agents such as benzyltriphenylphosphonium chloride (BTPPC)[8]; this treatment is not essential, however. The column was then conditioned by temperature programming at 0.2°/min to 250° with a helium flow-rate of 2 ml/min.

Polar column preparation. Columns were drawn from soft glass tubes (6 × 4 mm) and were etched with dry HCl as described by Alexander and Rutten[16]. After etching for 2 h at 350°, the glass turned opaque. For coating, the method described above was used. Using methylene chloride as solvent, the coating of a 17 m × 0.22 mm column (No. 9) at 24° required 18 h. The column was then conditioned by temperature programming at 0.1°/min to 235° with a helium flow-rate of about 2 ml/min.

Gas chromatograph. Columns were used in Carlo Erba Model GI-452 or 2300 gas chromatographs equipped with flame ionization detectors (FID). Chro-

matograms were recorded using simultaneously a 1-mV Servo-Riter II recorder. (Texas Instruments) and an Infotronics CRS 104 electronic digital integrator. The hydrogen flow was maintained constant by including a flow controller in the flow line.

Installation of capillary columns in the gas chromatograph. Two injection devices were used: an all-glass solid injector (Ros system[20]) and an all-glass liquid injector (see Fig. 1). Slight modifications were made to the former, consisting of an extension of the Pyrex tube (10 × 4 mm) through the injector block by means of a narrow-bore glass tube (6 × 1 mm). The column was positioned in this tube a few millimetres below the tip of the glass needle in the injection position. The column was fixed to the injector device by a reducer adapter (6 mm to 2 mm).

The all-glass liquid system is also shown in Fig. 1. The pre-column was a silanized Pyrex tube (6 × 3 mm) packed with 2% OV-101. The capillary column was gently pushed into the plug of glass-wool through a reducer (6 mm to 2 mm). The venting line was a Pyrex tube (6 × 3 mm). The stainless-steel buffer volume (100 ml) delayed the entry of the rejected sample into the flow meter so that the viscosity changes did not have an adverse effect on sample splitting and minimized

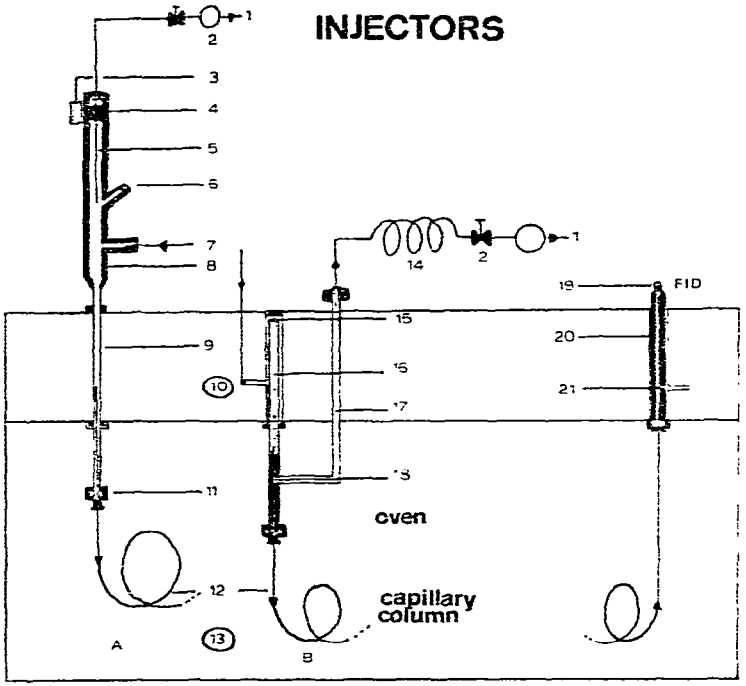


Fig. 1. Scheme for the installation of the capillary column into the gas chromatograph: A, All-glass solid injector; B, all-glass liquid injector; C, FID. 1 = Leak to atmosphere; 2 = flow-rate regulator; 3 = magnet; 4 = iron plunger; 5 = glass needle; 6 = injection septum; 7 = carrier gas inlet; 8 = glass tube (10 × 4 mm); 9 = glass tube (6 × 1 mm); 10 = injector block; 11 = reducer (6 mm to 2 mm); 12 = capillary column; 13 = oven; 14 = buffer, volume 100 ml; 15 = glass-wool; 16 = pre-column (OV-101, 2%); 17 = venting line; 18 = splitting zone; 19 = FID quartz jet; 20 = glass insert; 21 = hydrogen inlet.

contamination of the lines, the needle valve and the flow meter. This injector was a slightly modified version of that described by German and Horning[21].

The outlet capillary column ended 1 mm below the quartz jet of the FID. In this system, the dead volume from the column inlet to the detector was virtually zero. This simple equipment permitted columns to be changed quickly and simply.

Gas chromatographic conditions. The conditions used were: injector and detector temperatures 260°, column inlet pressure 0.5 bar, hydrogen flow-rate 10 ml/min, air flow-rate 150 ml/min and oven temperature about 220–250° in the isothermal mode.

In the all-glass solid injector, the leak to atmosphere was regulated at 20 ml/min and the helium flow-rate into the capillary column was about 1–2 ml/min. In the all-glass liquid injector, the splitting ratio was about 3:1 or 4:1, allowing a flow-rate of 1.5 ml/min into the column.

Derivative formation. TMS, HFB and TFA derivatives were prepared according to previously described methods[3]. The steroid derivatives were dissolved in *n*-hexane or carbon disulphide for injection.

Separation of human urinary dihydroxysteroids. The urinary $C_{19}O_2$ and $C_{21}O_2$ steroids were extracted and purified according to the methods currently used in this laboratory[22,23]. The extracts were resolved by thin-layer chromatography (TLC) on silica gel F_{254} in methylene dichloride–ethyl acetate (6:4) into two zones: a "5α zone", containing dihydroxysteroids with the 5α structure, and a "5β zone", containing dihydroxysteroids with the 5β structure.

This very time-consuming method was simplified by using liquid chromatography on a lipophilic gel.

Gel chromatography

Hydroxyalkylpropyl derivatives of Sephadex LH-20 gel (25–100 μm; Pharmacia, Uppsala, Sweden) were prepared by the method of Ellingboe *et al.*[24] using Nedox 1114 (A.D.M. Chemicals, Minneapolis, Minn., U.S.A.). Columns (50 cm × 1 cm I.D.) were packed by gravity with lipophilic gel containing 54% (w/w) of hydroxyalkyl residues in benzene as solvent.

Samples were dissolved in 1–4 ml of benzene and applied to the top of the gel bed. Approximate values for the standard elution volume (S.E.V., *i.e.*, the elution volume relative to the column bed volume multiplied by 100)[25] were measured by collecting 1-ml fractions. Compounds in these fractions were detected and quantified by GLC after derivative formation.

After this preliminary study, gel chromatography was applied to the purification of urinary C_{19} and C_{21} dihydroxysteroids using columns of bed volume 20 ml. Urinary extracts after hydrolysis and extraction were applied to the top of the gel bed in 4 ml of benzene. Fractions of S.E.V. 150–275 (30–55 ml) were collected and, after separation by TLC into 5α and 5β zones, were analyzed by GLC. The columns were regenerated by passing successively 25 ml of benzene–isopropanol (3:1, v/v) and 80 ml of benzene through them.

RESULTS AND DISCUSSION

Performance of capillary columns

The qualities of the columns were conveniently compared by calculating the

"coating efficiency"[26], which is the ratio of the theoretically maximum possible plate number over the highest experimental plate number attainable. The usual expressions were utilized:

$$n_{exp.} = 5.54 \left(\frac{t_r}{w_h}\right)^2$$

$$HETP_{exp.} = \frac{L}{n_{exp.}}$$

$$HETP_{min., calc.} = r\sqrt{\frac{1 + 6k + 11k^2}{3(1 + k)^2}}$$

$$k = \frac{t'_r}{t_M} = \frac{t_r - t_M}{t_M}$$

$$\text{Coating efficiency} = 100 \cdot \frac{HETP_{min., calc.}}{HETP_{exp.}}$$

$$\beta = \frac{V_G}{V_L} \cdot d_p = \frac{r}{2d_F}$$

where t_r, t'_r and t_M are the retention time of the sample component, the corrected retention time measured from the "air peak" and the retention time of an inert component, respectively, L is the column length, r is the column radius, k is the capacity ratio of compounds, w_h is the peak width at half-height, V_G is the gaseous volume, V_L is the liquid volume, d_p is the stationary phase density and d_F is the film thickness.

The results are presented in Table I. The "coating efficiency" obtained by using the OV-101 columns coated according to the static procedure was about 45%. This value, measured for a di-HFB steroid, was very close to the values obtained by other workers[8,19].

Several methods have been used for calculating column efficiencies[26]. Artificially high values can be obtained by using test compounds with relatively short retention times such as alkanes. Thus, for column No. 7, the efficiency measured using *n*-hexacosane ($k = 8.40$) was 134,000 theoretical plates and the coating efficiency was 57%. Therefore, we prefer to determine the number of theoretical plates and the characteristics of the columns using 5α-androstane-3α,17β-di-HFB under conditions very similar to our operating conditions.

In column preparation, there are two main problems: (i) the catalytic activity of the glass walls, which is lower than that of metals but high enough to cause serious difficulties in the analysis of thermolabile solutes such as steroids, and (ii) the poor wettability of glass by stationary phases. The catalytic activity of Pyrex glass seems to be sufficiently small not to require any deactivation by silanization or with thermostable ionic surface-active agents. Silanization has no effect on the efficiency and behaviour of the columns towards steroids. Silanized columns deteriorated after several months of use as the film became degraded, resulting in a decrease in the number of theoretical plates but not asymmetry of the steroid peaks. According to Rutten and Luyten[8], the lifetime of apolar capillary columns is increased by silani-

TABLE I

PERFORMANCES OF CAPILLARY COLUMNS

Column properties	*OV-101*								*OV-225*	
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Length (m)	25	56	40	55	40	48	60	58	17	45
I.D. (mm)	0.25	0.41	0.39	0.37	0.39	0.37	0.29	0.30	0.22	0.38
Phase ratio, β	194	194	194	231	215	213	215	234	387	275
Film thickness (μm)	0.32	0.52	0.51	0.40	0.45	0.43	0.34	0.43	0.15	0.68
Capacity ratio, k	8.85	7.10	7.10	7.15	7.55	7.40	9.4	10.3	9.0	12.5
Theoretical plate number	45,000	60,000	53,000	76,000	55,000	65,000	105,000	109,000	38,000	110,000
HETP (mm)	0.55	0.93	0.75	0.72	0.72	0.74	0.55	0.53	0.44	0.41
Coating efficiency (%)	40	39	45	44	47	45	47	51	46	83
R_{3-20} TMS	0.80	1.00	0.80	1.10	0.90	1.00	1.40	—	—	—
Glass	Pyrex	Pyrex	Pyrex	Pyrex	Pyrex	Pyrex	Pyrex	Soda-lime	Soda-lime	Soda-lime
Etched by HCl	No	No	No	No	No	No	No	Yes	Yes	Yes
Silanized	No	No	No	Yes	Yes	Yes	Yes	No	No	No
Deactivated	No	No	No	No	No	BPTC	BPTC	No		

zation. Deactivation by a surface-active agent such as BTPPC had no evident effect on the efficiency and lifetime of the columns.

The preparation process used for OV-225 seemed to be reproducible. It can be stated that columns coated with a polar phase are at least as efficient as OV-101 columns. The high coating efficiency with OV-225 (column No. 10) was noteworthy.

An attempt to chromatograph non-derivatized androstanediols was successful. The chromatograms did not show any tailing or asymmetry.

We were unable to prepare OV-7 columns with the technique described by Bouche and Verzele[19]. The reasons for this failure are not obvious. Boggaerts *et al.*[27] have discussed these difficulties for squalane, DC-550 and DC-220 stationary phases. We think that all of these phases have too low a viscosity (less than 500 cS).

Stability of columns

The columns were used routinely for the separation of human urinary steroids at 230–250°. After 3–4 months, the performances were unchanged, except for the capacity ratio, k. For column No. 4, the capacity ratio of 5α-androstane-3α,17β-diheptafluorobutyrate decreased by 6% in 4 months at 250° and the film thickness decreased from 0.50 to about 0.48 μm.

The repeated injection of a 2-μl sample volume with a splitting ratio between 3:1 and 4:1 did not destroy the capillary columns. We think that the use of carbon disulphide as the injection solvent may have contributed to the maintenance of column characteristics. The removal of solvent tailing with carbon disulphide was noteworthy.

In our hands, the behaviour of soda-lime glass capillary columns coated with OV-101 towards polar steroids such as 5β-pregnane-3α,17α,20α-triol-3,20-di-TMS became unsatisfactory after a few weeks of use. For instance, Fig. 2 shows the chromatographic peak of this compound on column No. 8. The asymmetry of this peak is probably due to the polar properties of the free 17α-hydroxyl group. Meanwhile, the behaviour of this column is very good towards such fully silylated steroids. This phenomenon was not observed when using Pyrex glass columns.

In Table I, it can be seen that the film thickness of OV-225 in column No. 9 was very small. In 3 weeks, the capacity ratio of 5α-androstane-3α,17β-di-TMS decreased by about 36% and the film thickness decreased from 0.15 to about 0.09 μm. With such a film thickness, it was likely that droplets of stationary phase would appear after a few days. Bouche and Verzele[19] reported that the maximum allowable

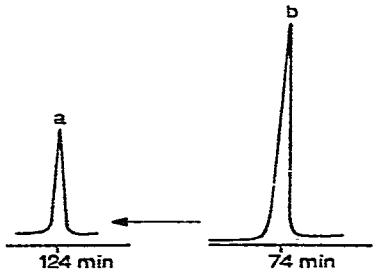


Fig. 2. Peak shapes for 5β-pregnane-3α,17α,20α-triol-3,20-di-TMS on OV-101 soda-lime glass column. (a) Immediately after conditioning; (b) after 2 weeks' operation at 250°.

operating temperature (MAOT) was much lower for glass capillary columns than for packed columns. The MAOT of OV-225 capillary columns was about 230° and this low value could explain the limited lifetime of column No. 9.

The performance of column No. 10 was maintained for about 1 month. After this period, the glass turned opaque, indicating total stationary phase bleeding. Many attempts to obtain a thermostable OV-225 capillary column failed. It was found that, in spite of recent advances[18,28], many key problems in the preparation of highly efficient and thermostable glass capillary columns have not been solved satisfactorily. Thus, surface phenomena associated with the poor wettability of glass are now of interest in the preparation of polar chromatographic columns[17,29–32].

Quality tests with capillary columns

The first measurement made on a new column was the determination of the optimum practical gas velocity[26]. Our experience has shown that this measurement can be shortened by using steroid pairs that are difficult to separate. On OV-101, two pairs can be used: 5α-androstane-3β,17β- and androst-5-ene-3β,17β-di-TMS (peaks 3 and 20) (see Fig. 3), and 5α-androstane-3β,17α- and 5β-androstane-3α,17β-di-HFB. The optimum operating conditions were determined when the maximum resolution was attained. The high-efficiency OV-101 column enabled us to separate two steroids having a relative retention value of $\alpha = 1.022$ ($\Delta I = 5$) with a peak resolution of 1.10 (see Fig. 3).

The quality test with the OV-225 capillary column could be made by injecting the pair 5α-androstane-3β,17α ($I = 2699$) and 5β-androstane-3α,17β ($I = 2703$) as the di-TFA derivatives or the pair 5α-androstane-3β,17β ($I = 2730$) and 5α-pregnane-3α,20β ($I = 2737$) as the di-HFB derivatives. Column No. 10 enabled us to separate two steroids (peaks 6 and 4) having a relative retention value of $\alpha = 1.035$ ($\Delta I = 4$) with a peak resolution of 1.30 (see Fig. 3).

Injection systems

The high resolving power of open-hole capillary columns has not been used extensively in the steroid field. One of the reasons why this technique is not common in steroid analysis is the difficulty of injection.

In this work, two injection systems were utilized: an all-glass solid system and an all-glass liquid system. In these two systems, contact of the sample with hot catalytically active surfaces is avoided. These two injectors have proved to be satisfactory for use with steroids (Fig. 3). The following advantages can be recognized for the solid injector: the reduction of the solvent peak, permitting the injection of large volumes; the maintenance of column performance; the absence of discrimination with respect to molecular size; and the accuracy and reproducibility. The disadvantages are: the non-versatility (gas and volatile products cannot be injected successfully) and the inferior operator convenience.

The liquid injector, which provides complete sample evaporation before the splitter zone, gives the advantages of injection on the top of a packed column: accuracy, reproducibility, and good operator convenience. With correct pre-column flow-rates (greater than 5 ml/min), the column performances were maintained at the optimum. The life of capillary columns was not shortened, owing to the absence of "solvent shock"[21]. The linearity of the splitter was studied by using a hydrocarbon

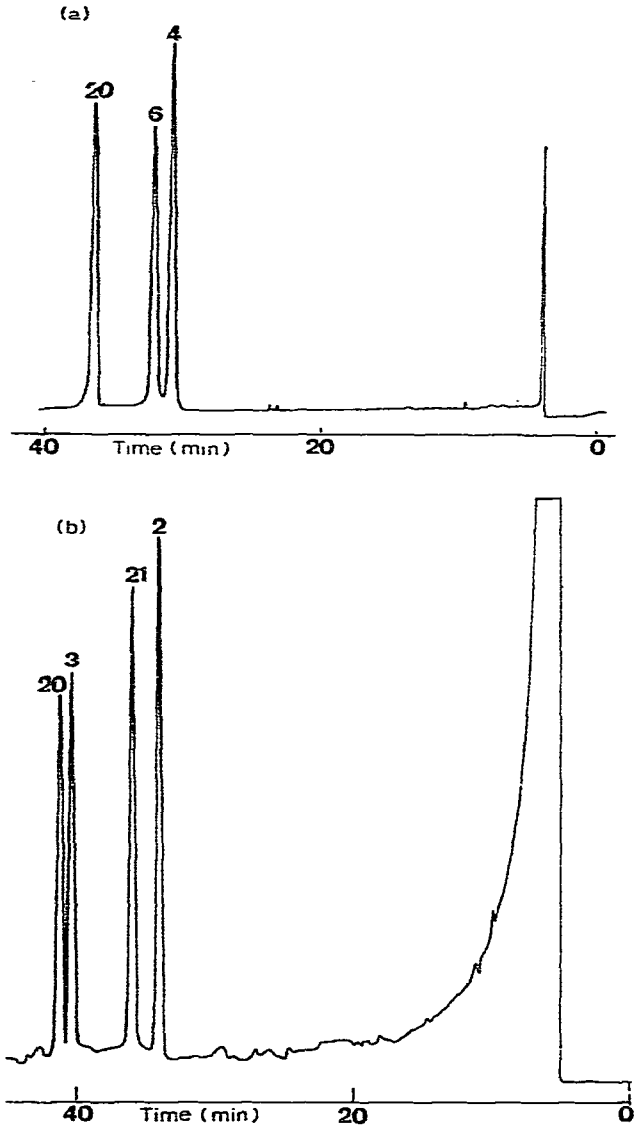


Fig. 3. Quality tests with capillary columns. The optimum operating conditions are attained when the resolution between peaks 3–20 and 4–6 is maximized. The peaks are numbered as in Table III. (a) OV-225, column No. 10; $R_{4-6} = 1.30$; $\alpha = 1.035$; TFA. (b) OV-101, column No. 4; $R_{3-20} = 1.10$; $\alpha = 1.022$; TMS.

mixture covering the range of retention indices in which the steroids studied were eluted. The ratios of the peak areas of *n*-alkanes were determined and compared with the ratios found by quantitative GC analysis with a packed column. The relative deviations from the packed column value are shown in Table II. In the range C_{22}–C_{32}, the linearity was acceptable; the results were in good agreement with those published by German and Horning[21]. The linearity was also studied by using a standard steroid

TABLE II

RELATIVE VARIATIONS IN RATIO OF PEAK AREAS OBSERVED FOR *n*-ALKANES AND TMS, TFA AND HFB STEROID DERIVATIVES BETWEEN ANALYSIS WITH A PACKED COLUMN AND WITH A CAPILLARY COLUMN EQUIPPED WITH AN ALL-GLASS LIQUID INJECTOR

Δ^5A = androst-5-ene; 5α-A = 5α-androstane; 5α-Pr = 5α-pregnane. Results given are percentages.

Group of compounds	*Compound pairs*	*Derivative*			
		None	*TMS*	*TFA*	*HFB*
n-Alkanes	C_{22}/C_{24}	−2.7	—	—	—
	C_{22}/C_{26}	−3.3	—	—	—
	C_{22}/C_{28}	−3.4	—	—	—
	C_{22}/C_{32}	+3.8	—	—	—
Steroids	Δ^5A-3β,17β/5α-Pr-3α,20α	—	−19	−174	−310
	5α-A-3α,17β/Δ^5A-3α,17α	—	+ 7.8	− 2.4	—
	5α-A-3α,17β/Δ^5A-3β,17β	—	+ 6.1	− 23.4	− 47.4
	5α-A-3α,17β/5α-Pr-3α,20α	—	−12	− 63	−126

mixture after TMS, TFA and HFB derivatization. The non-linearity (or selectivity) can be seen in Table II and increased from TMS to TFA and HFB derivatives. The reasons for these difficulties are unknown to us. Nevertheless, the TMS derivatives were acceptable for quantitative work, but the TFA and HFB derivatives were utilized only for qualitative work.

Retention indices of dihydroxysteroids

Kováts retention indices were determined using hydrocarbon standards. The retention indices of 33 dihydroxysteroids on OV-101 packed and capillary columns and on an OV-225 capillary column are given in Table III.

Good agreement was found between the retention indices on the OV-101 packed and capillary columns. The packed column gave slightly lower indices for the TMS and the TFA derivatives. Correlations between the retention indices obtained on the different types of column were highly significant: $r = 0.974$, 0.999 and 0.999, for the TMS, TFA and HFB derivatives, respectively. Consequently, routine identifications on open-tubular columns could be made by using retention indices measured on packed columns after slight corrections[15,33].

Fig. 4 shows a schematic representation of the retention indices on OV-225 and OV-101 columns. It can be seen that (i) the most specific derivatives that permitted a clear separation of dihydroxysteroids on OV-101 or OV-225 were HFB derivatives and (ii) two analyses on OV-225 and OV-101 permitted the complete resolution of 21 androstane(ene)- and pregnane(ene)-diols.

Steroidal allylic alcohols and their ethers or esters (TMS, TFA, HFB) were thermally degraded in GC. They showed two or many peaks with retentions significantly smaller than those expected from comparison with other unsaturated alcohols. Many workers[34,35] have shown that the peaks obtained resulted from the formation of the homoannular and heteroannular dienes.

ΔI values, defined as $\Delta I = I$ (OV-225) − I (OV-101), are shown in Table IV. The ester derivatives (HFB and TFA) were retained more on OV-225 than on OV-101;

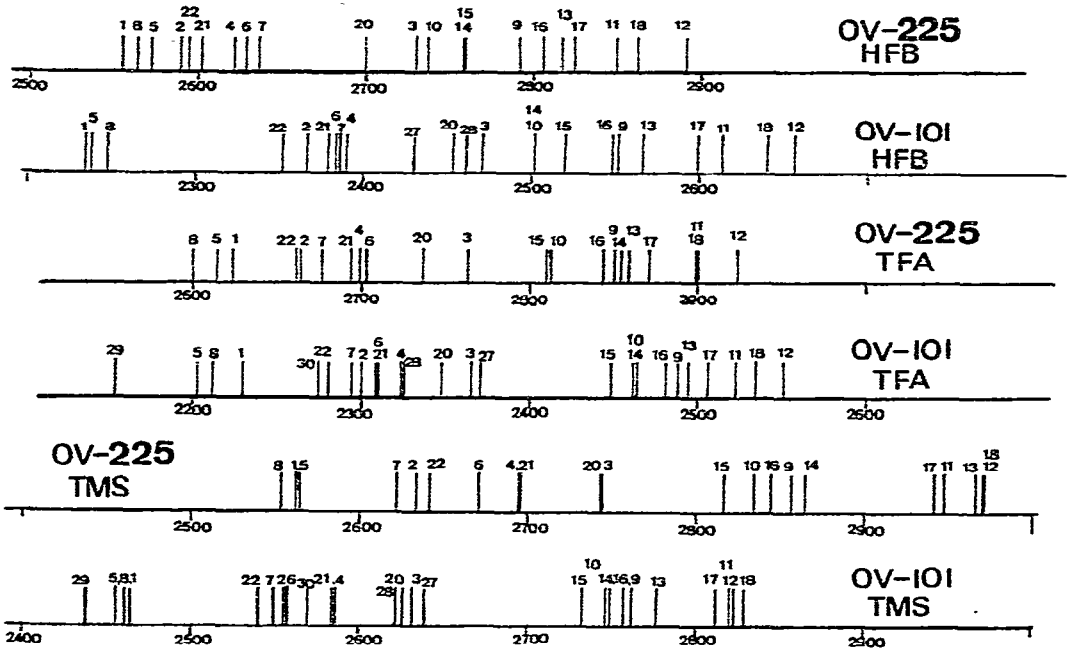


Fig. 4. Schematic representation of retention data for C_{19} and C_{21} dihydroxysteroids. The steroids are numbered as in Table III.

indeed the OV-225 phase is specific for keto and ester groups. The ΔI values of TMS derivatives were very variable (between 46 and 173). On the contrary, however, the ΔI values of TFA derivatives were virtually constant at about 377. The ΔI values of HFB compounds were also virtually constant at about 238, except for three 17α-hydroxysteroids. The abnormal behaviour of these 17α-hydroxylated compounds could be explained by the relative distance between the two heptafluorobutyrate groups. The ΔI value can therefore be a supplementary parameter in difficult identifications when a standard compound is not available.

The maximum standard deviation of the retention index for the same column and instrument for consecutive series of determinations on a few days was about 0.1%. Considering the small differences (1–5 index units) in the Kováts index values, absolute identification by GC could be difficult and required repeated analyses. For example, five analyses of urinary 5β-androstane-3α,17β-diol gave retention indices of 2572, 2578, 2573, 2577 and 2572; the standard compound gave a mean retention index of 2574.

Gel chromatography

Gel chromatography on dextran-modified lipophilic gels has been shown[25,36] to be dependent on the number of hydroxyl functions in the molecule with non-polar solvents such as benzene. The group separation of steroids according to the number of hydroxyl groups is therefore feasible. Table V shows the S.E.V. values of some steroids used in calibration. As shown, the steroids are eluted according to the number

TABLE III

RETENTION INDICES OF 33 DIHYDROXYSTEROIDS ON PACKED AND CAPILLARY COLUMNS

Compounds degraded by GC into two peaks (2), three peaks (3) and four peaks (4).

No.	*Steroids*	*Packed column (OV-101, 1.69%, 2.10 m × 4 mm)*			*Capillary column (OV-101, 55 m × 0.37 mm)*			*Capillary column (OV-225, 17 m × 0.225 mm)*		
		TMS (207°)	*TFA (207°)*	*HFB (214°)*	*TMS (252°)*	*TFA (236°)*	*HFB (236°)*	*TMS (193°)*	*TFA (193°)*	*HFB (187°)*
1	5α-A-3α,17α	2464	2222	2235	2476	2230	2235	2562	2623	2555
2	5α-A-3α,17β	2554	2287	2363	2575	2301	2367	2633	2664	2589
3	5α-A-3β,17β	2631	2350	2465	2655	2366	2471	2742	2763	2730
4	5α-A-3β,17α	2585	2310	2389	2604	2324	2390	2693	2699	2622
5	5β-A-3α,17α	2453	2196	2239	2444	2203	2238	2566	2614	2572
6	5β-A-3α,17β	2558	2296	2383	2572	2309	2384	2670	2703	2628
7	5β-A-3β,17β	2545	2279	2380	2570	2295	2386	2621	2677	2637
8	5β-A-3β,17α	2460	2203	2248	2474	2212	2248	2552	2599	2563
9	5α-PG-3α,20α	2764	2472	2548	2784	2488	2552	2856	2851	2792
10	5α-PG-3α,20β	2746	2449	2500	2767	2464	2502	2833	2813	2737
11	5α-PG-3β,20β	2820	2504	2607	2839	2523	2616	2946	2898	2849
12	5α-PG-3β,20α	2823	—	2652	2848	2551	2657	2970	2921	2891
13	5β-PG-3α,20α	2777	2482	2567	2794	2495	2567	2967	2859	2817
14	5β-PG-3α,20β	2749	2449	2502	2666	2462	2502	2864	2854	2758
15	5β-PG-3β,20β	2732	2432	2516	27541	2449	2520	2816	2811	2758
16	5β-PG-3β,20α	2757	2464	2568	2780	2482	2573	2843	2844	2806
17	PG-5-3β,20β	2812	2489	2593	2826	2507	2599	2941	2872	2824
18	PG-5-3β,20α	2831	2517	2636	2855	2535	2641	2971	2899	2862
19	PG-4-3β,20β	(4)	(4)	(4)	(3)	(4)	—	(2)	(2)	(2)
20	A-5-3β,17β	2625	2331	2449	2650	2348	2453	2737	2737	2699
21	A-5-3β,17α	2582	2298	2378	2600	2310	2379	2694	2694	2602
22	A-5-3α,17β	2538	2267	2347	2561	2281	2352	2663	2663	2594
23	A-4-3α,17α	2444	(3)	(4)	(3)	(3)	—	(2)	(2)	(3)
24	A-4-3α,17β	2542	(4)	(4)	(3)	(3)	—	2673	(3)	(2)
25	A-4-3β,17β	2614	(4)	(4)	(3)	(3)	—	(2)	(4)	(3)
26	A-4-3β,17α	2555	(4)	(4)	(3)	(3)	—	(2)	2484	(3)
27	5α-A-3β,16β	2640	2362	2462	2631	2369	2465	—	—	—
28	5α-A-3β,16α	2631	2344	2444	2623	2349	2430	—	—	—
29	5α-A-7α,17β	2437	2154	2201	2430	2158	2203	—	—	—
30	5α-A-7β,17β	2569	2269	2340	2568	2274	2363	—	—	—
31	5α-A-9(11)ene-3α,17β	2552	2265	2349	2544	2270	2320	—	—	—
32	5α-A-1-ene-3β,17β	2652	2364	—	2642	2368	(4)	—	—	—
33	A-diene-5-9(11)-3β,17β	2618	2301	2407	2615	2301	2406	—	—	—

TABLE IV

VARIATIONS IN RETENTION INDICES (I) BETWEEN OV-101 AND OV-225
$\Delta I = I$ (OV-225) $- I$ (OV-101).

Steroid	*TMS*		*TFA*		*HFB*	
	ΔI	$\Delta I \cdot 10^{-2}$ / I (OV-101)	ΔI	$\Delta I \cdot 10^{-2}$ / I (OV-101)	ΔI	$\Delta I \cdot 10^{-2}$ / I (OV-101)
1	86	3.47	393	17.6	320	14.3
2	58	2.25	363	15.7	222	9.4
3	87	3.27	397	16.7	259	10.4
4	99	3.80	375	16.1	232	9.7
5	122	5.00	411	18.6	334	14.9
6	98	3.81	394	17.0	246	10.2
7	51	1.98	382	16.6	251	10.5
8	78	3.15	387	17.5	315	14.0
9	72	2.58	363	14.6	240	9.4
10	46	1.66	349	14.2	235	9.4
11	107	3.77	375	14.8	235	9.0
12	122	4.28	373	14.6	234	8.8
13	173	6.19	364	14.6	250	9.7
14	98	3.54	392	15.9	256	10.4
15	62	2.25	362	14.8	238	9.4
16	63	2.23	362	14.6	233	9.0
17	115	4.07	365	14.6	225	8.6
18	116	4.07	364	14.3	221	8.3
20	92	3.47	389	16.6	246	10.0
21	95	3.65	384	16.6	223	9.3
22	88	3.43	382	16.7	242	10.2

of hydroxyl groups contained in the molecules. The ketonic groups are not retarded on the lipophilic gels. This chromatographic behaviour can be explained by solute adsorption on the gel by means of hydrogen bonds between the ether linkage of the gel network and suitable hydrogen donors in the solute, such as the hydroxyl groups[37,38].

Some epimeric steroids were completely or partially resolved by the lipophic gel. The separation between the axial (3α) and the equatorial (3β) hydroxylated epimers of the 5α-steroids was noteworthy. 5α-Androstane-3α,17β-diol and its 3β-epimer were resolved with a factor resolution about 0.80 with a column having an efficiency about 500 theoretical plates (HETP 0.5 mm). Commercial lipophilic gel (unsieved) has been reported to give an identical HETP. In order to obtain a better efficiency, the sieving of Sephadex LH-20 is necessary.

The separation between the androst-5-ene compounds and the corresponding dihydro-5α-steroids did not occur on the Nedox gel. On the other hand, the 3α- and the 3β-hydroxylated epimers of the steroids containing a double bond at the 5-position were completely resolved.

The type of separation obtained with this lipophilic gel column indicates that the technique is complementary to high-resolution gas chromatography.

TABLE V

ELUTION DATA FOR STANDARD STEROIDS ON THE SEPHADEX LH-20–NEDOX 1114 (54%) IN THE BENZENE SYSTEM

Results are expressed as S.E.V. values.

Steroid	*Approximate S.E.V. value*	*Fraction*
5α-Androstane-3,11,17-trione	65	Trione
5α-Androstane-3,7,17-trione	65	
5α-Androstane-3,17-dione	67	Dione
3,5-Cycloandrostane-6,7-dione	65	
Androst-4-ene-3,17-dione	65	
Pregn-4-ene-3,20-dione	64	
3β-Hydroxy-5α-Androstane-7,17-dione	95	Ol-dione
3α-Hydroxy-5α-Androstane-11,17-dione	90	
3α-Hydroxy-5β-Androstane-11,17-dione	95	
11β-Hydroxy-4-androstene-3,17-dione	108	
3α-Hydroxy-5α-Androstane-17-one	93	Ol-one
3α-Hydroxy-5β-Androstane-17-one	95	
17β-Hydroxy-5α-Androstane-3-one	111	
17β-Hydroxy-androst-4-ene-3-one	120	
3β-Hydroxy-androst-5-ene-17-one	125	
6α,17β-Dihydroxy-5α-androstane-3-one	150	Diol-one
3α,11β-Dihydroxy-5α-androstane-17-one	165	
3β,11β-Dihydroxy-5α-androstane-17-one	190	
5-Androstene-3α,17β-diol	135	Diol
3,5-Cycloandrostane-6β,17β-diol	145	
5α-Androstane-3α,17β-diol	230	Androstane(ene)
5α-Androstane-3β,17β-diol	260	
5β-Androstane-3α,17β-diol	250	
Androst-5-ene-3β,17α-diol	250	
Androst-5-ene-3β,17β-diol	255	
5α-Pregnane-3β,20β-diol	220	
5α-Pregnane-3α,20α-diol	185	Pregnane(ene)
5β-Pregnane-3α,20α-diol	215	
Pregn-4-ene-3β,20α-diol	185	
Pregn-4-ene-3β,20β-diol	170	
5β-Pregnane-3β,17α,20α-triol	280	Triol
5β-Pregnane-3α,17α,20α-triol	380	
Pregn-5-ene-3β,17α,20α-triol	380	
5α-Androstane-3α,11β,17β-triol	470	
5α-Androstane-3α,11β,17β-triol	560	
5α-Androstane-3β,7β,17β-triol	560	

Application to the identification, separation and measurement of human urinary dihydroxysteroids

In a previous paper[3], we described the difficulties encountered in the quantification of urinary androstane(ene)- and pregnane-diols by means of packed column GC. The simultaneous measurement of 5α-androstane-3α- and -3β,17β-diols required an epoxidation reaction that allowed the elimination of interfering androst-5-ene-

3β,17β-diol. The high-efficiency open-tubular glass columns allowed the resolution of 5α-androstane- and androst-5-ene-3β,17β-diols (Fig. 3).

Human C_{19} and C_{21} dihydroxysteroids were extracted and purified according to the following method. A one-tenth aliquot of 24-h urines was incubated with *Helix pomatia* juice at 37° and the free steroids were extracted with diethyl ether. The dihydroxysteroids were purified by chromatography on lipophilic gel and fractionated by TLC[3] into two zones of polarity 5α and 5β. These two zones were analyzed by GLC on an OV-101 capillary column after TMS formation. Quantification was achieved by using as the internal standard androst-5-ene-3β,16β,17β-tri-TMS (I = 2884) or 5β-pregnane-3α,17α,20α-triol-3,20-di-TMS (I = 2940).

The identification of urinary dihydroxysteroids is based on three criteria: (i) behaviour identical with that of the reference standards during the different purification steps (lipophilic gel chromatography and TLC), (ii) retention indices on capillary columns identical with those of the corresponding standards and (iii) characteristic fragments in mass spectra identical with those of the reference compounds and no significant additional peaks in the high mass region[39,40].

Fig. 5 shows the gas chromatograms of 5α polarity male urine extract after TMS, TFA and HFB derivatization. Many compounds appeared to be isopolar with 5α-androstane-3α,17β-diol (peak No. 2). Peaks 2, 21, 20, 3, 9, 18 and 4 were identified on the basis of Kováts retention indices (Table VI). The identification was confirmed by mass spectrometry[40].

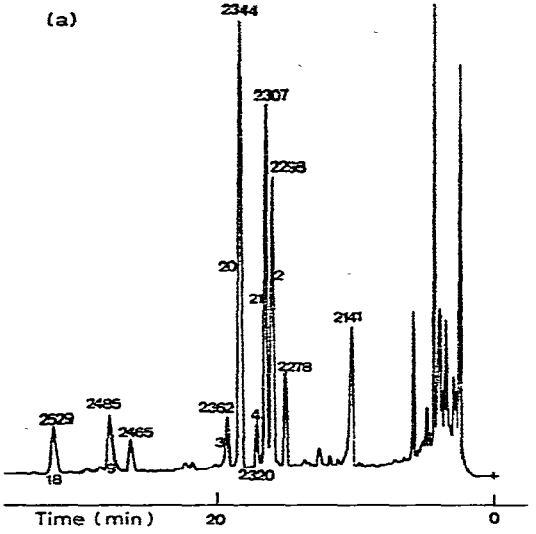
As shown in Fig. 6, the 5β polarity male urine extract contained two main peaks (6 and 13), which were identified as 5β-androstane-3α,17β-diol and 5β-pregnane-3α,20α-diol, respectively. When the reliable purification on lipophilic gel was used, two supplementary peaks appeared at I = 2658 (11β,3α-dihydroxy-5α-androstane-17-one) and I = 2665 (11β,3α-dihydroxy-5β-androstane-17-one). The minor shoulder of peak 6 has not yet been identified; preliminary results suggest that this compound has an unsaturated nucleus.

The direct quantitative analysis of C_{19} and C_{21} dihydroxysteroids by GLC was impossible, for several reasons such as the complexity of the steroid fraction from the biological mixtures and the low levels of these steroids compared with other compounds with similar structures such as 3α-hydroxy-5α-androstane-17-one. It therefore appeared necessary to carry out preliminary purifications by chemical methods such as Girard's reaction or by alumina chromatography. In order to simplify the previously described method[3] using these procedures, it seemed interesting to associate two methods: group fractionation by lipophilic gel chromatography and the high efficiency of capillary columns.

Purification by hydrophobic gel chromatography appeared to be a reliable procedure (see Table VII); in particular it was less time consuming than the previous method. This type of chromatography has many advantages, *e.g.*, high load capacity, high recovery (95–100%), chemical inertness, group fractionation without separation of individuals and clean blanks.

The specificity of the method was enhanced by using open-tubular columns. This allowed particularly the measurement of 5α-androstane-3β,17β-diol (peak 3), which was difficult to resolve from the compound unsaturated at position 5 (ref. 3). Two androstanediols, metabolites of testosterone, could be measured simultaneously.

The use of capillary columns enhances the detection sensitivity. The minimum


(a)
2344
2307
2258
20
2
21
2141
2278
2529
2485
2362
2465
4
3
18
9
2320
Time (min)
20
0

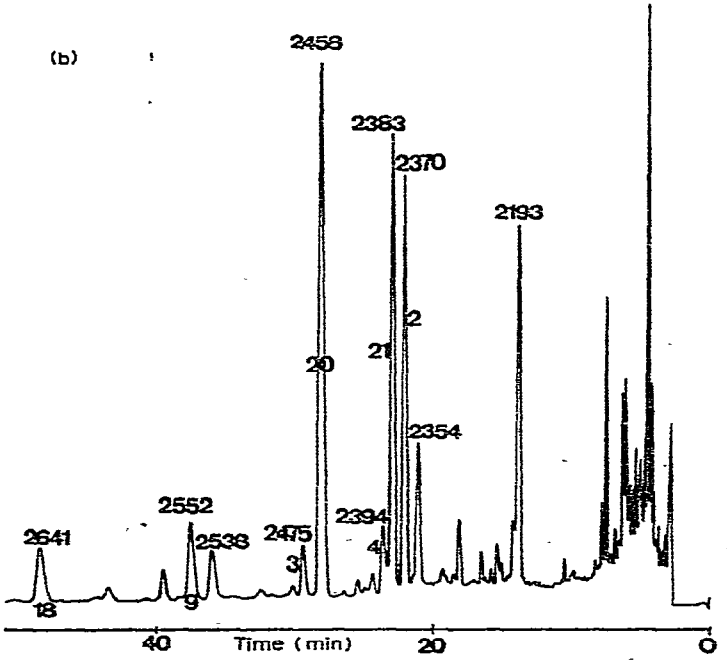

(b)
2456
2383
2370
2193
2
21
20
2354
2552
2394
2641
2538
2475
4
3
18
9
40
Time (min)
20
0

Fig. 5.

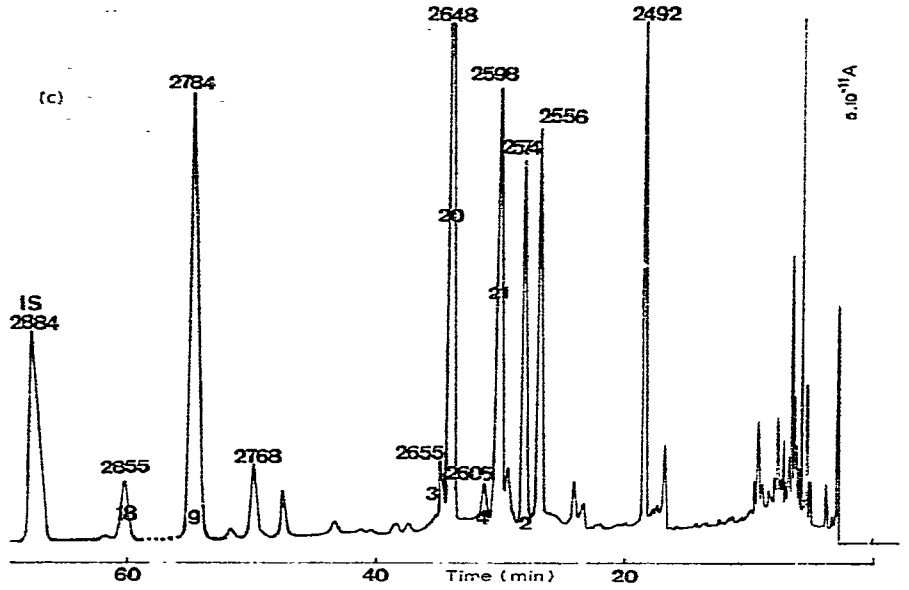


Fig. 5. Gas chromatograms of male urine extract. (a) TFA; (b) HFB; (c) TMS derivatives; 5α polarity. Column: (a) OV-101, 236°; (b) OV-101, 236°; (c) OV-101 column No. 3, 250°. The peaks are numbered as in Table III.

TABLE VI

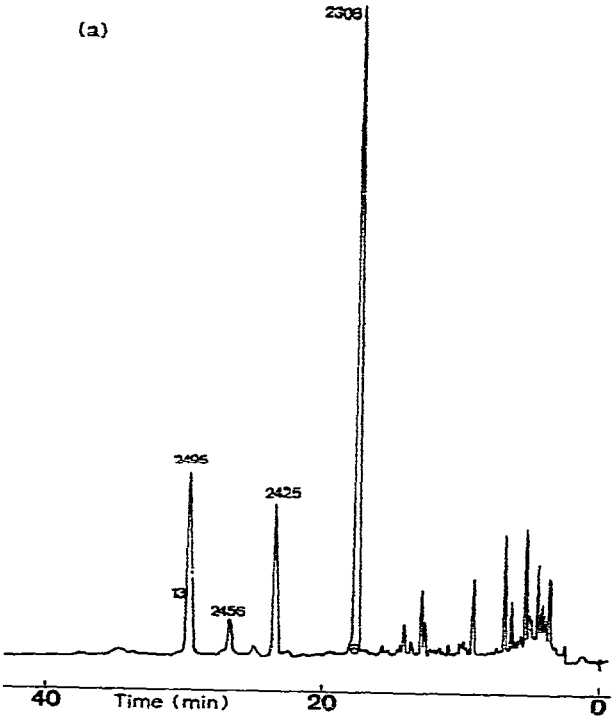
RETENTION INDICES OF URINARY AND STANDARD COMPOUNDS, NUMBERED AS IN TABLE III

Peak No.	*OV-101*						*OV-225*	
	TMS		*TFA*		*HFB*		*TMS*	
	Urinary	*Standard*	*Urinary*	*Standard*	*Urinary*	*Standard*	*Urinary*	*Standard*
2	2574	2575	2298	2301	2370	2367	2633	2633
21	2598	2600	2307	2310	2383	2379	2694	2695
20	2648	2650	2344	2348	2458	2453	2742	2737
3	2655	2655	2362	2366	2475	2471	2742	2742
9	2784	2784	2485	2488	2552	2552	2853	2856
18	2855	2855	2529	2535	2641	2641	2969	2971
4	2605	2604	2320	2324	2394	2390	2695	2693
6	2572	2572	2308	2309	2385	2384	2671	2670
13	2797	2794	2495	2495	2569	2567	2965	2967

concentration which can be assessed by the method is 1 μg per 24 h for 5α-androstane-3α,17β-diol. This limit can be reduced by injecting more than 2 μl in the solid system injector.

The coefficient of variation was determined from duplicate and triplicate analyses for two concentration ranges of 5α-androstanediol-3α,17β, the values obtained being for 15% 0–50 μg per 24 h and 5% for 50–300 μg per 24 h.

In eight determinations of urine from normal males aged 25–40 years, the mean excretion of 5α-androstane-3α,17β-diol was 80 ± 23 (s.d.) μg per 24 h and of


(a)
2308
2496
2425
13
2456
40
Time (min)
20
0

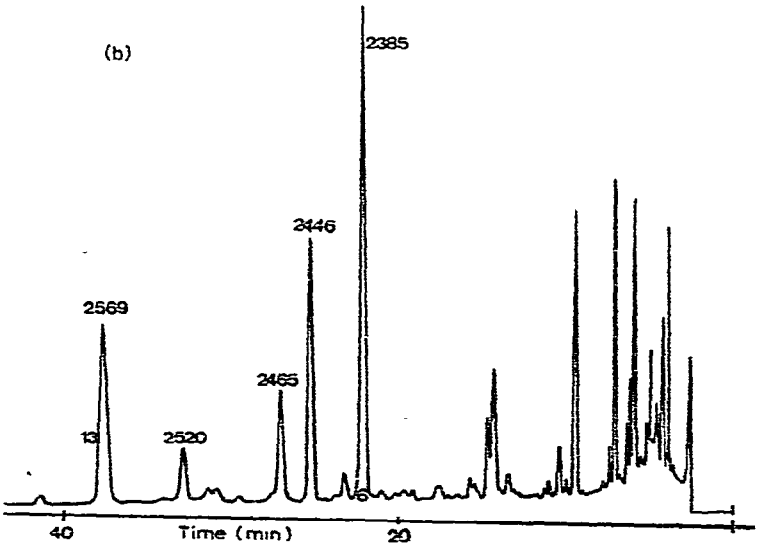

(b)
2385
2446
2569
2465
13
2520
40
Time (min)
20

Fig. 6.

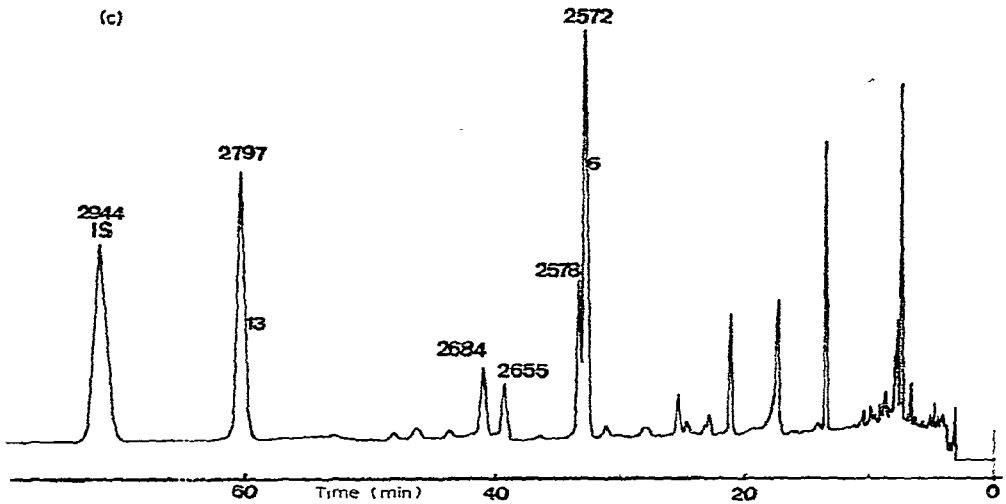


Fig. 6. Gas chromatograms of male urine extract. (a) HFB; (b) TFA; (c) TMS derivatives; 5β polarity. Column: (a) OV-101, 236°; (b) OV-101, 236°; (c) OV-101 column No. 5, 238°. The peaks are numbered as in Table III.

TABLE VII

RELIABILITY AND REPRODUCIBILITY OF METHOD INCLUDING PURIFICATION BY LIPOPHILIC GEL CHROMATOGRAPHY

Results are expressed as μg per 24 h. Each value represents the average of five determinations on the same urine.

Steroid	*Purification by lipophilic gel*	*Purification according to ref. 3*
5α-Androstane-3α,17β-diol	211 ± 10	205 ± 12
5α-Androstane-3β,17β-diol	42 ± 2	42 ± 2
Androst-5-ene-3β,17α-diol	197 ± 8	190 ± 10
Androst-5-ene-3β,17β-diol	324 ± 12	347 ± 14
5α-Pregnane-3α,20α-diol	63 ± 5	58 ± 6
5β-Androstane-3α,17β-diol	329 ± 8	319 ± 10
5β-Pregnane-3α,20α-diol	534 ± 8	528 ± 8

5α-androstane-3β,17β-diol 26 ± 5 (s.d.) μg per 24 h. In eight determinations of urine from normal females aged 20–30 years, the corresponding values were respectively 32 ± 8 (s.d.) μg per 24 h and 11 ± 6 (s.d.) μg per 24 h. The 5α-androstane-3α,17β-diol originates from the metabolism of testosterone in target tissues to androgens[41] and is an index of androgenicity. The physiological significance and mechanism of action of the 3β-diol epimer are not yet well established. We think that the specific quantification of these epimers in diagnosis and stimulation or suppression tests should help future investigations in human pathology.

CONCLUSION

The combination of lipophilic gel chromatography and high-efficiency glass capillary column chromatography provides an important means of identification and

quantification of urinary metabolites. The former system gives a group fractionation according to the number of hydroxyl functions in the molecule without the separation of individuals components. The latter system gives the high efficiency necessary to resolve many isomers.

An interesting development in the use of glass open-tubular columns coupled with gel liquid chromatography is the rapid identification and measurement of metabolites from androgen steroids. Further, preliminary results suggest that this method may be useful in diagnosis and stimulation or suppression tests in clinical pathology.

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REFERENCES

1 E. C. Horning, W. J. A. VandenHeuvel and B. G. Creech, in D. Glick (Editor), *Methods in Biomedical Analysis*, Vol. 11, Interscience, New York, 1963, p. 69.
2 M. G. Horning, E. C. Chambaz, C. J. Brooks, A. M. Moss, E. A. Boucher, E. C. Horning and R. M. Hill, *Anal. Biochem.*, 31 (1969) 512.
3 F. Berthou, L. Bardou and H. H. Floch, *J. Chromatogr.*, 93 (1974) 149.
4 K. Grob, *Helv. Chim. Acta*, 51 (1968) 51.
5 K. Grob and G. Grob, *J. Chromatogr. Sci.*, 7 (1969) 584.
6 M. Novotny and K. Tesarik, *Chromatographia*, 1 (1968) 332.
7 A. L. German, C. D. Pfaffenberger, J. P. Thenot, M. G. Horning and E. C. Horning, *Anal. Chem.*, 45 (1973) 930.
8 G. A. F. M. Rutten and J. A. Luyten, *J. Chromatogr.*, 74 (1972) 177.
9 J. A. Völlmin, *Chromatographia*, 3 (1970) 233.
10 J. A. Völlmin and H. H. Curtius, *Z. Klin. Chem. Klin. Biochem.*, 9 (1971) 43.
11 J. A. Völlmin, *Clin. Chim. Acta*, 34 (1971) 207.
12 M. Novotný and A. Zlatkis, *J. Chromatogr. Sci.*, 8 (1970) 346.
13 M. Novotný and A. Zlatkis, *J. Chromatogr.*, 56 (1971) 353.
14 A. Ros and I. F. Sommerville, *J. Obstet. Gynaecol. Br. Commonw.*, 78 (1971) 1096.
15 A. L. German and E. C. Horning, *J. Chromatogr. Sci.*, 11 (1973) 76.
16 G. Alexander and G. A. F. M. Rutten, *Chromatographia*, 6 (1973) 231.
17 M. Novotný and K. Grohmann, *J. Chromatogr.*, 84 (1973) 167.
18 P. Vanhout, J. Szafranek, C D. Pfaffenberger and E. C. Horning, *J. Chromatogr.*, 99 (1974) 103.
19 J. Bouche and M. Verzele, *J. Gas Chromatogr.*, 13 (1968) 501.
20 A. Ros, *Monit. Obst.-Ginecol. Endocrinol. Metab.*, 39 (1968) 777.
21 A. L. German and E. C. Horning, *Anal. Lett.*, 5 (1972) 619.
22 F. L. Berthou, L. G. Bardou and H. H. Floch, *J. Steroid Biochem.*, 2 (1971) 141.
23 F. Berthou, L. Bardou and H. H. Floch, *J. Steroid Biochem.*, 3 (1972) 819.
24 J. Ellingboe, E. Nystrom and J. Sjövall, *J. Lipid Res.*, 11 (1970) 266.
25 C. J. W. Brooks and R. A. B. Keates, *J. Chromatogr.*, 44 (1969) 509.
26 L. S. Ettre, *Open Tubular Columns in Gas Chromatography*, Plenum Press, New York, 1965, pp. 18 and 22.
27 T. Boggaerts, M. Verstappe and M. Verzele, *J. Chromatogr. Sci.*, 10 (1972) 217.
28 G. Alexander, G. Garzó and G. Pályi, *J. Chromatogr.*, 91 (1974) 25.
29 M. Nečasová and K. Tesařík, *J. Chromatogr.*, 79 (1973) 15.
30 K. D. Bartle and M. Novotný, *J. Chromatogr.*, 94 (1974) 35.
31 G. Alexander and G. A. F. M. Rutten, *J. Chromatogr.*, 99 (1974) 81.
32 M. Gassiot-Matas, J. O. Pascual-Calveras and A. Serra-Macia, *Chromatographia*, 5 (1972) 328.

33 J. A. Luyten, *Thesis, Eindhoven*, 1973, p. 114.
34 R. A. Okerholm, P. Bretcher and H. H. Wotiz, *Steroids*, 12 (1968) 435.
35 A. A. Kandutsch, *Steroids*, 10 (1967) 31.
36 R. A. Anderson, B. A. Knights and C. J. W. Brooks, *J. Chromatogr.*, 82 (1973) 337.
37 R. A. Anderson, G. Defaye, C. Madani, E. M. Chambaz and C. J. W. Brooks, *J. Chromatogr.*, 99 (1974) 485.
38 R. A. Anderson, E. M. Chambaz, G. Defaye, C. Madani, T. A. Baillie and C. J. W. Brooks, *J. Chromatogr. Sci.*, 12 (1974) 636.
39 F. L. Berthou, D. Picart, L. G. Bardou and H. H. Floch, *J. Chromatogr. Sci.*, 12 (1974) 662.
40 D. Picart, *Thèse 3ème Cycle*, Université de Brest, Brest, 1975.
41 N. Bruchovsky and J. D. Wilson, *J. Biol. Chem.*, 243 (1968) 2012.