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APPLICATION OF OVER-RUN THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY IN THE SEPARATION OF CLOSELY RELATED $C_{19}O_2$ STEROIDS

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SUMMARY

An improved method for the separation of epimeric $C_{19}O_2$ steroids and their related allylic alcohols is described. In this method, the steroids are first separated by over-run thin-layer chromatography, and the unresolved groups are further analysed as free or as trimethylsilyl ether derivatives by gas–liquid chromatography. The behaviour of twenty-one $C_{19}O_2$ steroids was investigated by thin-layer chromatography in four systems and by gas–liquid chromatography in four liquid phases. All steroid pairs of similar polarity were resolved by the combination of these two fractionation procedures.

INTRODUCTION

In spite of the development of new analytical procedures, the resolution of several pairs of steroids belonging to the $C_{19}O_2$ series remains difficult. Neither by application of thin-layer chromatography (TLC) with multiple development[1] nor by gas–liquid chromatography (GLC)[2] is it possible satisfactorily to separate closely related steroids of this group, such as the epimeric androstanediols or hydroxyandrostanones, from their parent allylic alcohols. The isolation of allylic steroids as metabolites from testosterone and androstenedione in several biological materials[3–10] makes their separation from the parent saturated compounds necessary.

In view of the importance of this question, experiments were undertaken to separate and identify 3,17-dioxygenated C_{19} steroids. The combination of continuous TLC with GLC offers a means for solving this problem, and the results obtained for twenty-one steroids are presented in this paper.

EXPERIMENTAL

Reagents

1,1,1,3,3,3-Hexamethyldisilazane was purchased from Eastman Kodak (Rochester, N.Y., U.S.A.); all other reagents and solvents were from E. Merck (Darmstadt, G.F.R.).

Steroids

5α-Androstane-3α (and 3β), 17α-diols were obtained from Schering (Berlin, G.F.R.). The 3α- and 3β-hydroxyandrost-4-en-17-one were prepared by reduction of androst-4-ene-3,17-dione with sodium tetrahydroborate in isopropanol at room temperature overnight, as indicated by Kupfer[11], whereas the androst-4-ene-3α (and 3β), 17β-diols were obtained by reduction of 1 mg of androst-4-ene-3,17-dione and 17β-hydroxyandrost-4-en-3-one with 5 mg of sodium tetrahydroborate for 15 min in methanol–water (4:1) at 37°. The reaction products were separated by TLC, and their purity was assayed by GLC. All other steroids were obtained from Steraloids (Flushing, N.Y., U.S.A.).

Thin-layer chromatography

Ascending-development continuous TLC (over-run TLC) was performed on 0.25-mm layers of silica gel $60F_{254}$ (Merck) in two ways: (a) by additionally covering a 4-cm-wide zone at the top of the plate with a slurry prepared by shaking 12 g of silica gel G–calcium sulphate (7:3) with 20 ml of methanol–water (1:1), the prepared layer being dried overnight at room temperature; and (b) by using a metal container as described by Züllich *et al.*[12]. This container had an angle of 45° to the layer and was filled with dry silica gel G; a 1-mm space between the layer and the container provided better solvent transfer from the layer into the silica gel in the container. In each system, the chromatogram was developed in saturated conditions (closed tanks) until the solvent front had risen to the upper part of the layer (60 min); the tank was then opened 1.5 cm; the over-run time began at this moment. For instance, 2-h continuous development signifies 1 h normal run with the tank closed followed by 2 h over-run.

Solvent systems

Four solvent systems were used for continuous development of free steroids: S-1, cyclohexane–ethyl acetate (1:1) (2 h over-run); S-2, *n*-hexane–ethyl acetate (3:1) (90 min); S-3, cyclohexane–ethyl acetate (4:1) (90 min); and S-4, benzene–ethyl acetate (1:1) (90 min). For TLC of the acetates, S-3 (90 min) was used.

Detection of spots

The spots were made visible by the reaction of Ekkert [0.5% anisaldehyde solution in sulphuric acid–acetic acid (49:1)]. Allylic alcohols were detected by the reaction of Winogradow (trichloracetic acid). Details of the tests have been described[1].

Gas–liquid chromatography

A Pye gas chromatograph, Series 104 (Pye Unicam, Cambridge, Great Britain), equipped with a dual flame ionization detector and coiled glass columns (2.7 m × 4 mm) was used. The columns were conditioned for 48 h with an argon or nitrogen carrier gas flow-rate of 26 to 35 ml/min at an inlet pressure of 3 atm. and a temperature programme of 1°/min from 100 to 250°. The flash-heater temperature was 260° and the detector temperature was 250°. The following stationary phases were used, each coated on Gas-Chrom Q (100–120 mesh; 150–125 μm) and obtained from Werner Günther Analysentechnik (Düsseldorf, G.F.R.): 2.2% (w/w) of SE-30 (temperature

225°; retention time of cholestane 25.6 min); 3% (w/w) of QF-1 (205°; 11.3 min); 1% (w/w) of XE-60 (190°; 12.4 min); and 3% (w/w) of OV-225 (225°; 20 min).

Formation of derivatives

The acetates were prepared under a nitrogen atmosphere overnight by adding 0.5 ml of pyridine–acetic anhydride (1:1) to the dry steroid; after complete evaporation, each acetate was dissolved in ethanol and spotted on to the silica gel layer.

The trimethylsilyl (TMS) ethers were prepared by addition of 0.05 ml of hexamethyldisilazane and 0.01 ml of trimethylchlorosilane to the steroid dissolved in 1 ml of anhydrous pyridine. After 30 min at 60°, the reaction mixture was evaporated, resuspended in hexane and centrifuged briefly to precipitate ammonium chloride; the supernatant solution containing the TMS ether was used for GLC. All the samples were injected in 1 to 3 μl of hexane.

RESULTS AND DISCUSSION

Table I shows the mobility values found by over-run TLC for $C_{19}O_2$ steroids

TABLE I

BEHAVIOUR OF $C_{19}O_2$ STEROIDS AND THEIR ACETATES ON SILICA GEL G BY ASCENDING OVER-RUN TLC IN SEVERAL SOLVENT SYSTEMS

The solvents S-1, S-2, S-3 and S-4 were as defined in the text; mobilities of the steroids are expressed in cm: development was for up to 2.5 h.

Steroid[*]	*Free steroid*				*Acetate*
	S-1	*S-2*	*S-3*	*S-4*	*S-3*
5α-A-3,17-dione	13.6	8.5	—	15.3	—
5β-A-3,17-dione	12.8	7.2	—	14.9	—
4-A-3,17-dione	9.0	4.0	2.3	12.5	—
17β-hydroxy-5α-A-3-one	10.6	5.0	2.9	11.8	8.0
17β-hydroxy-5β-A-3-one	8.7	3.2	2.0	—	7.6
17β-hydroxy-4-A-3-one	6.7	2.3	1.4	8.7	5.0
17α-hydroxy-4-A-3-one	6.7	—	—	—	—
3α-hydroxy-4-A-17-one	8.1	—	—	9.2	—
3β-hydroxy-4-A-17-one	10.2	—	—	11.5	—
3α-hydroxy-5α-A-17-one	10.3	4.7	2.7	11.2	9.3
3β-hydroxy-5α-A-17-one	9.0	4.0	2.4	—	9.5
3α-hydroxy-5β-A-17-one	7.7	—	1.6	8.7	9.8
3β-hydroxy-5β-A-17-one	10.9	—	3.2	11.9	9.3
5α-A-3α,17β-diol	7.9	3.2	—	—	—
5α-A-3β,17β-diol	7.6	2.9	—	8.2	—
5β-A-3α,17β-diol	4.7	1.2	—	5.3	—
5β-A-3β,17β-diol	9.0	3.7	—	9.4	—
5α-A-3α,17α-diol	5.7	—	—	5.8	—
5α-A-3β,17α-diol	7.0	—	—	—	—
4-A-3α,17β-diol	5.6	1.6	—	—	—
4-A-3β,17β-diol	8.5	3.0	—	—	—
5-A-3β,17β-diol	8.1	—	—	—	—
5-A-3β,17α-diol	8.1	—	—	—	—

[*] A = androstane or androstene.

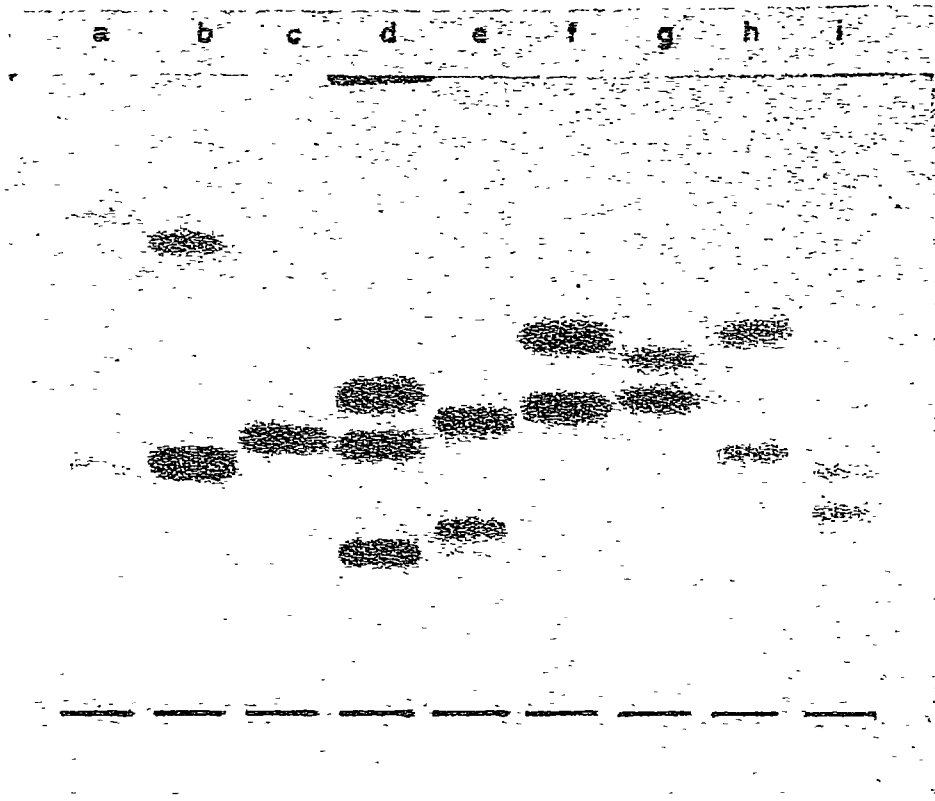

Fig. 1. Separation of closely related $C_{19}O_2$ steroids by over-run TLC on silica gel G developed with cyclohexane–ethyl acetate (1:1); after 1 h of development under saturation conditions, the lid of the tank is partly opened (1.5 cm) and development is continued for 90 min; the upper edge of the layer was evenly thickened by application of 12 g of silica gel G–calcium sulphate (7:3). Standards: (a) 5α-androstane-3,17-dione (top), androst-4-ene-3,17-dione (middle) and 17β-hydroxyandrost-4-en-3-one (below); (b) 5β-androstane-3,17-dione (less polar) and 17β-hydroxyandrost-4-en-3-one (more polar); (c) 5α-androstane-3α,17β-diol; (d) 5β-androstane-3β,17β-diol (top), 5α-androstane-3β,17β-diol (middle) and 5β-androstane-3α,17β-diol (below); (e) androst-4-ene-3β,17β-diol (less polar) and androst-4-ene-3α,17β-diol (more polar); (f) 17β-hydroxy-5α-androstan-3-one (less polar) and 17β-hydroxy-5β-androstan-3-one (more polar); (g) 3α-hydroxy-5α-androstan-17-one (less polar) and 3β-hydroxy-5α-androstan-17-one (more polar); (h) 3β-hydroxy-5β-androstan-17-one (less polar) and 3α-hydroxy-5β-androstan-17-one (more polar); (i) 5α-androstane-3β,17α-diol (less polar) and 5α-androstane-3α,17α-diol (more polar).

in four solvent systems and for their acetate derivatives in one system. These values indicate that only for the three pairs of steroids testosterone–epitestosterone, androsterone–3β-hydroxyandrost-4-en-17-one and the 5α-androstane-3α, 17β- and -3β, 17β-diols resolution was not accomplished.

In Fig. 1 is reproduced a chromatogram of the continuous TLC of a group of mixtures of $C_{19}O_2$ steroids in system S-1. A comparison of these data with those obtained in multiple runs in the same solvent system indicates that optimum separation is achieved by continuous running. For instance, the migration values (in cm) for androstenedione, aetiocholanolone and androstanedione after three runs in system S-2 (ref. 13) were 3.2, 5.2 and 5.7, respectively, whereas after 150 min of

TABLE II

RETENTION TIMES (RELATIVE TO CHOLESTANE) OF $C_{19}O_2$ STEROIDS (FREE AND AS TMS DERIVATIVES) ON VARIOUS STATIONARY PHASES

For GLC conditions, see text.

*Steroid**	*Stationary phase*						
	SE-30		*QF-1*		*XE-60*		*OV-225*
	TMS	*Free*	*TMS*	*Free*	*TMS*	*Free*	*TMS*
5α-A-3,17-dione	—	0.46	—	4.50	—	2.31	4.20**
5β-A-3,17-dione	—	0.40	—	4.20	—	—	3.25**
4-A-3,17-dione	—	0.64	—	6.85	—	3.56	6.60**
17β-hydroxy-5α-A-3-one	0.57	0.96	1.78	2.72	1.11	2.07	1.45
17β-hydroxy-5β-A-3-one	0.51	0.88	1.62	2.46	0.99	1.89	1.29
17β-hydroxy-4-A-3-one	0.69	1.14	2.80	4.20	1.62	3.26	2.23
17α-hydroxy-4-A-3-one	—	—	2.37	—	—	—	1.84
3β-hydroxy-4-A-17-one	—	—	1.35	—	—	—	1.35
3α-hydroxy-4-A-17-one	—	—	1.06	—	—	—	—
3α-hydroxy-5α-A-17-one	0.44	0.86	1.09	2.04	0.72	1.56	0.92
3β-hydroxy-5α-A-17-one	0.54	0.88	1.51	2.33	1.01	1.79	1.35
3α-hydroxy-5β-A-17-one	0.43	0.80	1.20	2.16	0.82	1.58	1.08
3β-hydroxy-5β-A-17-one	0.40	0.79	1.11	1.86	0.72	1.41	0.92
5α-A-3α,17β-diol	0.52	0.48	0.47	1.21	0.53	1.42	0.39
5α-A-3β,17β-diol	0.63	0.43	0.63	1.37	0.61	1.58	0.57
5β-A-3α,17β-diol	0.52	0.38	0.48	1.26	0.52	1.40	0.44
5β-A-3β,17β-diol	0.50	0.37	0.47	1.10	0.50	1.28	0.39
5α-A-3α,17α-diol	0.39	0.38	0.37	1.18	0.43	1.38	0.30
5α-A-3β,17α-diol	0.56	0.42	0.52	1.34	0.57	1.55	0.50
4-A-3α,17β-diol	0.49	0.26	0.44	1.21	0.51	0.50	0.44
4-A-3β,17β-diol	0.60	0.27	0.59	1.26	0.60	0.52	0.58

* A = androstane or androstene.

** As free steroid.

continuous TLC, the migration values were 4.0, 7.2 and 8.5. However, multiple runs in different systems sometimes allow more effective separation of closely related steroids. Epimeric androstanediols have been completely resolved[13] after two runs in ethyl acetate–cyclohexane (1:1), followed by a development in benzene–ethanol (9:1), the mobilities (in cm) being 7.8 for 5β-A-3α.17β-diol*, 9.6 for 5α-A-3β,17β-diol, 10.6 for 5α-A-3α,17β-diol and 11.2 for 5β-A-3β,17β-diol; in continuous TLC, 5α-A-3α,17β-diol and 5α-A-3β,17β-diol were not resolved.

Retention times relative to cholestane obtained during GLC on four stationary phases for a series of $C_{19}O_2$ androstane and androst-4-ene steroids are summarized in Table II. The steroid pairs that remained unresolved after over-run TLC were satisfactorily separated on several of the phases. The separation of androsterone and 3β-hydroxyandrost-4-en-17-one is, for instance, best accomplished on OV-225 or QF-1; with the latter phase, both steroids are also separable from epiandrosterone.

Several pairs of steroids remain unresolved, or are only partly resolved, by GLC. All these pairs, which often include an allylic alcohol, are satisfactorily separable by continuous TLC. This fact makes a combination of both these chromato-

* A = androstane.

TABLE III

SEPARATION OF PAIRS OF $C_{19}O_2$ STEROIDS BY COMBINATION OF OVER-RUN TLC AND GLC

*Steroid pair**	*Over-run-TLC*	*GLC (as TMS ether)*
5α-A-3,17-dione–5β-A-3,17-dione	S-2	OV-225
17β-hydroxy-5α-3-one–17β-hydroxy-5β-3-one	S-1, S-2	OV-225, QF-1
17β-hydroxy-4-A-3-one–17α-hydroxy-4-A-3-one	Unresolved**	OV-225, QF-1
3α-hydroxy-5α-A-17-one–3α-hydroxy-4-A-17-one	S-1	Unresolved
3β-hydroxy-5α-A-17-one–3β-hydroxy-4-A-17-one	S-1	QF-1
3α-hydroxy-5α-A-17-one–3β-hydroxy-5β-A-17-one	S-3	Unresolved
3β-hydroxy-4-A-17-one–3α-hydroxy-5α-A-17-one	Unresolved	QF-1, OV-225
3α-hydroxy-4-A-17-one–3α-hydroxy-5α-A-17-one	S-1	Resolution difficult
5α-A-3α,17β-diol–5α-A-3β,17β-diol	Unresolved	SE-30, QF-1, XE-60, OV-225
5β-A-3α,17β-diol–5β-A-3β,17β-diol	S-1, S-2	OV-225 (partial resolution)
5α-A-3α,17β-diol–4-A-3α,17β-diol	S-1, S-2	OV-225 (partial resolution)
5α-A-3β,17β-diol–4-A-3β,17β-diol	S-1, S-2	Unresolved

* A = androstane or androstene.

** Optimal resolution was obtained by over-run TLC on alumina with dichloromethane as developing solvent (J. Paul and B. P. Lisboa, unpublished results).

graphic methods highly effective for the resolution of $C_{19}O_2$ steroids of closely similar polarity. Table III gives a summary of these difficult pairs and the possibility of resolving them.

The combination of these two analytical procedures has been shown to be useful in the identification of small amounts of labelled metabolites in biological material[9,10,14]; thus, these compounds, sometimes formed only in traces, can be identified by their behaviour in paper chromatography, TLC and radio gas chromatography.

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