CHROMATOGRAPHIC SEPARATION OF C_{19}-16-DEHYDRO-STEROIDS

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Since the discovery of androst-16-en-3α-ol in male and female urine by BROOKSBANK AND HASLEWOOD[1] and of its biosynthesis in testis and adrenocortical tissue by GOWER AND HASLEWOOD[2] and GOWER[3], the need for chromatographic systems to separate this compound from other C_{19}-16-dehydro-steroids which might also occur naturally has become increasingly acute. Besides being all weakly polar, these compounds which have been used in the present study, only differ from one another by changes in configuration at the A/B ring junction or by one double bond, thus making their separation more difficult. This difficulty has already been appreciated by BAKER AND GOWER[4], when only partial success was obtained with gas chromatography using a silicone gum stationary phase. Column chromatography with Al_2O_3 as adsorbent has been employed for the initial purification of urinary or tissue extracts and separates androst-16-en-3α-ol but the group of closely related steroids, called the "androstenol analogues" by BROOKSBANK[5] and by GOWER[3], are eluted in one fraction.

In view of the success with which thin layer chromatography has been employed in the separation of weakly polar steroids by BARBIER, JÄGER, TOBIAS AND WYSS[6], by VAN DAM, DE KLEUVER AND DE HEUS[7] and by VAN DAM[8], this technique has been applied to the present problem. In addition, an investigation has been made of some of the paper chromatographic systems suggested by other workers for the separation of weakly polar steroids employing as stationary phase phenylcellosolve (NEHER AND WETTSTEIN[9]; RUBIN, DORFMAN AND PINCUS[10]), kerosene (MARTIN[11]) and liquid paraffin (KODICEK AND ASHBY[12]). Systems using papers which had been fully acetylated (RITTER AND HARTEL[13]) or impregnated with silicic acid (LEA, RHODES AND STOLL[14]) have also been investigated.

EXPERIMENTAL

The 16-dehydro steroids used in this study were 5α-androst-16-en-3α-ol (An (α)), 5α-androst-16-en-3β-ol (An (β)), 5β-androst-16-en-3α-ol (aetiochol-16-en-3α-ol, Ae), androsta-5, 16-dien-3β-ol (Andien). Oestra-1, 3, 5(10), 16-tetraen-3-ol (Oe), a C_{18} steroid, was used as a comparison. An (α) was prepared by BROOKSBANK AND HASLEWOOD[1] and the other compounds were generously supplied by Dr. C. L. Hewett of Organon Ltd. Acetates were prepared using pyridine and acetic anhydride at room temperature overnight and were recrystallised from aqueous ethanol. Solutions for chromatography of concentrations of about 1 mg/ml and 10 mg/ml were made in methanol.

Thin layer chromatography

Smooth glass plates (18 × 18 cm or 18 × 6 cm, thickness 0.4 cm) were normally used although, in preliminary experiments, the use of microscope slides allowed a large number of runs to be performed in a short time. Initially, plates were covered with Kieselgel G using a thin-layer applier[15] but since unevenness and slight irregularities of the layer were not found to influence greatly the R_F of the same compound applied at intervals across a plate, the spreading was performed by hand in later experiments. In this case, a fairly thin mixture of one part Kieselgel G to three parts of water was prepared. The consistency was such that the mixture could be poured on to the plates after which the latter were gently tilted so as to allow fairly uniform spreading. Plates so prepared were dried first in air for 30 min and then at 100° for 60 min. The initial slow drying process appeared to be essential to prevent cracking of the surface of the thin-layer during reactivation at 100°. The preparation and development of chromatostrips and chromatoplates using the ascending technique were as described previously[7].

On some occasions plates were re-run. When the solvent front had almost reached the end of the plate, it was removed from the solvent and the position of the front marked. The plate was then dried, replaced in the solvent and the vessel closed. When the solvent front had reached the pre-marked position, the plate was again removed and dried. This procedure was repeated three or four times, to allow an effective run of solvent of about 50 cm.

Paper chromatography

Whatman No. 3MM paper was impregnated with silicic acid by the method of LEA, RHODES AND STOLL[14]. Runs were performed using the ascending technique by dipping one end of the paper into a few cm depth of solvent contained in a glass jar or tank which was then closed. No period of equilibration was necessary.

Detection of steroids

The colour reaction of BROOKSBANK AND HASLEWOOD[1] has been used to detect the 16-dehydro-steroids and their acetates used in this study on chromatostrips, chromatoplates and on some paper chromatograms. The two reagents (resorcyaldehyde in glacial acetic acid (0.5% w/v) and concentrated sulphuric acid in glacial acetic acid (5% v/v) were mixed in equal volumes prior to spraying. This solution will be abbreviated to RA. Colour development at 100°, however, was largely inhibited by the presence of non-volatile, stationary phases remaining on paper chromatograms after drying and, moreover, the reagent could not be used on fully acetylated paper since the latter was attacked by sulphuric acid. Of more general use, therefore, was the phosphomolybdic acid reaction (PMA) of KRITCHEVSKY AND KIRK[16] although the presence of large quantities of phenylcellosolve partially inhibited colour development. The use of phosphotungstic acid (PTA) in ethanol[11], iodine in light petroleum[17] and ALLEN[18] reagent (concentrated sulphuric acid–ethyl alcohol–water (80:18:2, by vol.)) has also been investigated. A solution of uranyl nitrate (5% w/v) in 10% v/v aqueous sulphuric acid* has also been used to detect the steroids on plates although silicic acid-impregnated paper charred with it on heating.

* I am grateful to Dr. R. J. BRIDGWATER for drawing my attention to this reagent.

TABLE I

APPROXIMATE R_F VALUES OF SOME 16-DEHYDRO-STEROIDS AND THEIR ACETATES ON KIESELGEL G IN VARIOUS SOLVENTS AND SOLVENT MIXTURES

Compound*	Solvents**												Colours with visualising reagents***				
	10	11	12	13	14	15	16	17	18	19	20	21	PMA*	PTA*	RA*	Allen[20]	Uranyl nitrate
An (α)	0.08	0.29	0.21	0.34	0.24	0.33	0.66	0.40	0.75	0.61	0.74	0.52	bg	br	m	gr	gr
Ae	0.05	0.22	0.19	0.26	0.20	0.27	0.58	0.33	0.62	0.55	0.65	0.45	g	br	m	gr	bgr
An (β)	0.04	0.19	0.15	0.20	0.15	0.20	0.48	0.27	0.46	0.45	0.58	0.38	b	br	m	gr	p
Andien	0.04	0.18	0.16	0.23	0.17	0.23	0.49	0.28	0.49	0.47	0.59	0.40	p	ro	b	m	p
Oe	0.15	0.38	0.37	0.48	0.30	0.51	0.72	0.51	0.93	0.77	0.88	0.80	rp	o	r	pk	r
An (α) acetate	0.34	0.69	0.51	0.64	0.75	0.91	1.0	0.68	1.0	—	0.87	0.81	g	ybr	m	gr	pk
Ae acetate	0.36	0.75	0.52	0.63	0.74	0.93	1.0	0.70	1.0	0.85	0.87	0.81	g	ybr	m	gr	bgr
An (β) acetate	0.40	0.88	0.54	0.62	0.77	0.91	1.0	0.71	1.0	0.91	0.88	0.78	g	ybr	m	gr	p
Andien acetate	0.40	0.79	0.55	0.61	0.79	0.89	1.0	0.71	1.0	0.84	0.89	0.75	p	p	m	m	br
Oe acetate	0.43	0.79	0.53	0.64	0.80	0.94	1.0	0.72	1.0	0.85	0.87	0.78	m	or	r	pk	r

* For abbreviations see Experimental Section.

** Solvents; abbreviations: 10 = toluene; 11 = methylene chloride; 12 = toluene–ethyl acetate, 19:1 (v/v); 13 = toluene–ethyl acetate, 9:1 (v/v); 14 = benzene–ethanol, 98:2 (v/v); 15 = benzene–ether, 9:1 (v/v); 16 = benzene–acetone, 85:15 (v/v); 17 = benzene–methyl ethyl ketone, 9:1 (v/v); 18 = methylene chloride–ethyl acetate, 9:1 (v/v); 19 = ethylene chloride–ethyl acetate, 90:12 (v/v); 20 = di-isopropyl ether–acetic acid, 96:4 (v/v); 21 = di-isopropyl ether–formic acid, 99:1 (v/v).

*** Colours; abbreviations: b = blue; bg = bluish green; bgr = bluish grey; br = brown; g = green; gr = grey; m = mauve; o = orange; r = red; or = orange red; ro = rose; rp = reddish purple; p = purple; pk = pink; y = yellow; yb = yellow brown.

RESULTS

Thin layer chromatography

The R_F values of An (α) and other 16-dehydro-steroids have been determined in a number of solvents and solvent mixtures using Kieselgel G as adsorbent. These data, together with those obtained for the acetates, are given in Table I. An (α), Ae and Oe could be separated from each other and from An (β) and Andien in a number of systems. The latter pair of compounds, however, resisted all attempts at resolution. The effect on the resolution of this pair of compounds of re-running some systems up to three or four times was studied. Using this technique a separation sufficient for their identification in a mixture has been achieved. Differences in the mobility of An (α) and Ae have also been accentuated (Table II). It appeared that an effective

TABLE II

EFFECT OF RE-RUNNING ON THE SEPARATION OF SOME 16-DEHYDRO-STEROIDS

Solvent	*Effective distance moved by solvent front (cm)**	*Distance moved from origin by steroids** (cm)*				
		An (α)	*Ae*	*An (β)*	*Andien*	*Oe*
Benzene–methyl ethyl ketone, 9:1 (v/v)	15.0	6.0	4.95	4.1	4.2	7.65
	44.7	10.75	9.65	8.4	8.7	12.4
	64.0	12.0	11.0	9.5	10.1	14.2
Toluene–methyl ethyl ketone, 9:1 (v/v)	44.4	9.25	7.95	6.5	6.85	11.3
	58.0	11.2	10.15	8.75	9.0	12.8
Toluene–ethyl acetate, 9:1 (v/v)	15.6	5.2	4.1	3.25	3.55	7.4
	45.9	8.7	7.4	6.15	6.55	11.65
	65.6	10.5	9.15	7.35	7.75	13.55
Ethylene chloride–ethyl acetate, 90:12 (v/v)	14.7	8.9	8.1	6.85	7.25	11.3
	30.6	12.0	10.9	9.1	9.6	13.8

* Chromatography was performed on glass plates (18 cm × 18 cm) covered with Kieselgel G. Development was continued until the solvent front had reached a pre-marked line. The plate was then removed from the solvent, dried and re-run. This procedure was repeated until the solvent front had effectively moved three to four times the length of the plate.

** For abbreviations, see Experimental Section.

run of 40–50 cm gave the maximum resolution possible in, for example, benzene–methyl ethyl ketone (9:1, v/v) and that there was little advantage to be gained from further runs. A typical run for the solvent to move an effective distance of 3 × 16 cm, allowing time to dry the plate in between runs, took only 2–2½ h (Fig. 1).

Visualisation of steroids on chromatoplates

Oe and Andien and their acetates gave distinctive colours with PMA, PTA, RA and the Allen reagent. The colours developed with PMA, RA and the Allen reagent by heating the plates at 110° for about 4 min, but only Oe and Andien and their acetates gave colours with PTA in this time. The other steroids and particularly the acetates of An (α), Ae and An (β) required about 8 min heating at 110°. On standing in daylight for about 20 min., all the colours with PTA had faded to mauve except those of Oe and Oe acetate which turned red. Iodine in light petroleum was found useful since

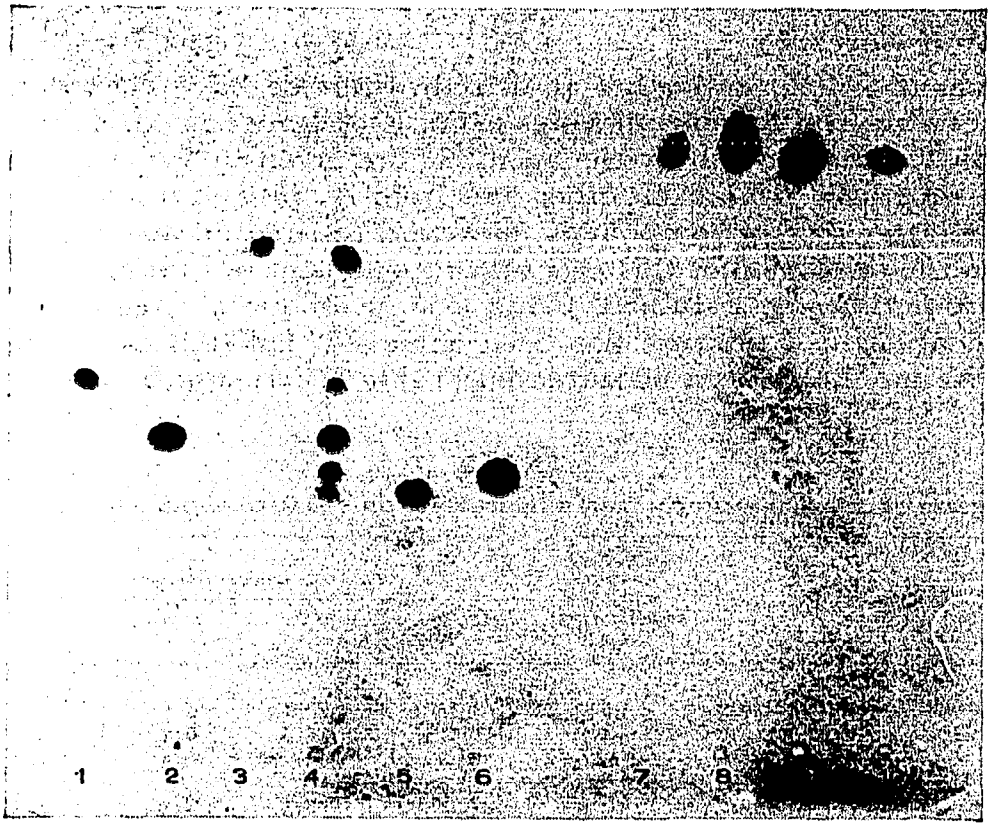

Fig. 1. Separation of 16-dehydro-steroids and some acetates on Kieselgel G using benzene–methyl ethyl ketone (9:1 v/v) as mobile phase. The plate was run three times allowing time for the solvent to evaporate between each run. 1 = 5α-androst-16-en-3α-ol; 2 and 7 = 5β-androst-16-en-3α-ol and acetate; 3 and 8 = oestra-1,3,5(10),16-tetraen-3-ol and acetate; 5 and 9 = 5α-androst-16-en-3β-ol and acetate; 6 and 10 = androsta-5,16-dien-3β-ol and acetate; 4 = mixture of 1, 2, 3, 5 and 6.

all the compounds gave yellow spots in the cold. The reaction appeared to be reversible since the spots faded completely after about 30 min. Most of the compounds gave specific colours with the uranyl nitrate reagent; 6–7 min heating at 110° was required (Table III).

Use of silicic acid-impregnated paper

The success with which phosphatides have been separated on silicic acid-impregnated paper in past years[14] suggested the possibility of applying this technique to the separation of C_{19}-16-dehydro-steroids and their acetates. The results obtained, summarized in Table III, were no better than those using the thin layer technique. Prolonged running of some systems overnight using the ascending technique greatly enhanced the separation of An (α) and Ae but An (β) and Andien still moved as one spot (Table IV).

The resolution of these steroids on fully acetylated paper or in systems using phenylcellosolve, kerosene and liquid paraffin as stationary phase, was disappointing. An (α) could be separated fairly easily from Ae, An (β) and Andien, but there was little difference, however, between the mobility of the androstenol analogues. Alteration of the mobile phase, the amount of stationary phase in the paper and overrunning of chromatograms produced no better results.

TABLE III

APPROXIMATE R_F VALUES OF SOME 16-DEHYDRO-STEROIDS AND THEIR ACETATES ON SILICIC ACID-IMPREGNATED PAPER

*Compound**	*Solvents***											*Colours with visualising reagents****			
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*PMA**	*PTA**	*RA**	*Allen*[20]
An (α)	0.17	0.47	0.52	0.38	0.33	0.61	0.59	0.63	0.42	0.30	0.91	bg	pk	m	m
Ae	0.10	0.36	0.42	0.30	0.25	0.53	0.47	0.55	0.34	0.21	0.88	bg	pk	m	m
An (β)	0.08	0.29	0.34	0.26	0.18	0.46	0.41	0.48	0.28	0.17	0.84	bg	pk	m	y
Andien	0.09	0.31	0.37	0.28	0.20	0.47	0.43	0.50	0.31	0.19	0.85	p	ro	b	m
Oe	0.24	0.64	0.69	0.51	0.46	0.77	0.71	0.74	0.55	0.40	0.95	m	o	or	ro
An (α) acetate	0.55	0.84	0.86	0.95	0.68	1.0	0.91	0.85	0.72	0.60	1.0	bg	pk	m	m
Ae acetate	0.61	0.85	0.87	0.96	0.69	1.0	0.92	0.87	0.72	0.64	1.0	bg	pk	m	m
An (β) acetate	0.60	0.85	0.88	0.95	0.70	1.0	0.92	0.87	0.71	0.65	1.0	bg	pk	m	y
Andien acetate	0.60	0.86	0.87	0.94	0.69	1.0	0.92	0.87	0.70	0.64	1.0	p	ro	b	m
Oe acetate	0.60	0.86	0.88	0.96	0.71	1.0	0.94	0.90	0.72	0.65	1.0	m	o	r	p

* For abbreviations see Experimental Section.

** Solvents; abbreviations: 1 = light petroleum (b.p. 83–97°)–benzene, 1:1 (v/v); 2 = benzene; 3 = benzene–ether, 99:1 (v/v); 4 = toluene–ether, 99:1 (v/v); 5 = toluene–ethyl acetate, 99:1 (v/v); 6 = ethylene chloride; 7 = benzene–methylene chloride, 50:50 (v/v); 8 = benzene–ethylene chloride, 50:50 (v/v); 9 = benzene–methyl ethyl ketone, 99:1 (v/v); 10 = toluene; 11 = methylene chloride.

*** Colours; abbreviations: b = blue; bg = bluish green; m = mauve; o = orange; r = red; or = orange red; ro = rose; p = purple; pk = pink y = yellow

TABLE IV

MOBILITY IN CM/HOUR OF SOME 16-DEHYDRO-STEROIDS RUN ON SILICIC ACID IMPREGNATED PAPER FOR 18 HOURS*

Compound**	Solvent		
	Light petrol–benzene 1 : 1 (v/v)	*Benzene*	*Benzene–ether 995 : 5 (v/v)*
An (α)	0.54	1.0	—
Ae	0.40	0.75	0.86
An (β)	0.26	0.62	0.70
Andien	0.26	0.62	0.70

* Chromatography was performed using the ascending technique. A pad of filter paper was clipped to the upper end of the chromatogram to absorb solvent.

** Abbreviations are given in Experimental Section.

DISCUSSION

The order of mobilities of the 16-dehydro-steroids (Oe > An (α) > Ae > Andien > An (β)) was constant in all the solvents used both on Kieselgel G and on silicic acid-impregnated paper. Resolutions were slightly better on the former due presumably to the compactness of the spots obtained. For practical purposes it was possible to separate Oe, An (α) and Ae from each other and from An (β) and Andien on thin layer plates if the overrunning technique was used. The difference in mobility between An (β) and Andien was too small, however, to allow anything better than a separation for the purposes of identification although Andien could be distinguished in a mixture by virtue of the specific colours given with PMA and RA.

The iodine reagent was useful in that all the compounds tested gave yellow spots in the cold. Moreover, the reaction was reversible so that a whole plate, consisting of marker lanes and unknown lanes, could be sprayed with no danger of some iodine spots remaining. It should be pointed out, however, that with iodine in vapour form, 10–20 μg quantities of all the compounds gave intense, yellow-brown spots which faded only slowly and incompletely so that if these were rechromatographed the yellow-brown, presumed iodo-derivatives remained at the origin (BROOKSBANK, unpublished). This could lead to serious errors in quantitative measurements.

The difficulty with which this series of 16-dehydro-steroids could be separated has confirmed conclusions previously made using gas chromatography[4] but improved resolution might be obtained using the trimethyl silyl ethers[19]. On theoretical grounds better separations should also be possible if a capillary column equivalent to 45,000 theoretical plates is employed[4]. Another approach to this problem makes use of the fact that when dehydro*epi*androsterone and *epi*androsterone, another pair of 3β-hydroxy-steroids which are difficult to separate[20] are oxidised, the corresponding 3,17-di-ketones can be resolved easily. Accordingly, experiments are being pursued to determine whether the 3-ketones corresponding to An (β) and Andien can be separated.

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SUMMARY

The chromatography of a closely related series of 16-dehydro-steroids and their acetates on thin layer plates and silicic acid-impregnated paper is described. Fairly rapid and easy separations of most of the compounds studied can be achieved although the separation of androst-16-en-3β-ol and androsta-5,16-dien-3β-ol is only obtained by an overrunning technique. Silicic acid-impregnated paper appears to be free from disadvantages associated with papers impregnated with other stationary phases such as phenylcellosolve, liquid paraffin and kerosene. The use of a number of colour reagents is described, including phosphomolybdic acid, phosphotungstic acid, resorcylaldehyde–sulphuric acid, the Allen reagent and uranyl nitrate in sulphuric acid. Some of the steroids give specific colours which may be useful in their identification.

NOTE ADDED IN PROOF

Since this paper was submitted excellent separations of the trimethyl silyl ethers of An (α), Ae, An (β) and Andien have been obtained by gas chromatography on a QF 1 column.

REFERENCES

1 B. W. L. Brooksbank and G. A. D. Haslewood, *Biochem. J.*, 80 (1961) 488.
2 D. B. Gower and G. A. D. Haslewood, *J. Endocrinol.*, 23 (1961) 253.
3 D. B. Gower, *J. Endocrinol.*, 26 (1963) 173.
4 R. W. R. Baker and D. B. Gower, *Nature*, 192 (1961) 1074.
5 B. W. L. Brooksbank, *J. Endocrinol.*, 24 (1962) 435.
6 M. Barbier, H. Jäger, H. Tobias and E. Wyss, *Helv. Chim. Acta*, 42 (1959) 2440.
7 M. J. D. van Dam, G. J. De Kleuver and J. G. De Heus, *J. Chromatog.*, 4 (1960) 26.
8 M. Van Dam, *Bull. Soc. Chim. Belg.*, 70 (1961) 122.
9 R. Neher and A. Wettstein, *Helv. Chim. Acta*, 35 (1952) 276.
10 B. L. Rubin, R. I. Dorfman and G. Pincus, *J. Biol. Chem.*, 203 (1953) 629.
11 R. P. Martin, *Biochim. Biophys. Acta*, 25 (1957) 408.
12 E. Kodicek and D. R. Ashby, *Biochem. J.*, 57 (1954) xii.
13 F. J. Ritter and J. Hartel, *J. Chromatog.*, 1 (1958) 461.
14 C. H. Lea, D. N. Rhodes and R. D. Stoll, *Biochem. J.*, 60 (1955) 353.
15 E. Stahl, *Chemiker-Ztg.*, 82 (1958) 323; *Parfuem. Kosmetik*, 39 (1958) 564.
16 D. Kritchevsky and M. R. Kirk, *Arch. Biochem. Biophys.*, 35 (1952) 346.
17 I. E. Bush, *Nature*, 166 (1950) 445.
18 W. M. Allen, S. J. Hayward and A. Pinto, *J. Clin. Endocrinol. Metab.*, 10 (1950) 54.
19 M. A. Kirschner and M. B. Lipsett, *J. Clin. Endocrinol. Metab.*, 23 (1963) 255.
20 S. H. Weinmann, O. Crepy, E. E. Baulieu, J. Guy and M. F. Jayle, *Bull. Soc. Chim. Biol.*, 39 (1957) 463.