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COMPARISON OF ADSORPTION AND REVERSED-PHASE PARTITION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF ANDROGENS

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SUMMARY

Sixty-nine androstane derivatives were chromatographed by adsorption and reversed-phase high-performance liquid chromatography (HPLC). The total number of hydroxyl and keto groups on the androstane molecule is the most important factor in determining their chromatographic behavior. An α,β-unsaturated keto group contributes as much to the polarity of the molecule as a hydroxyl group and more than an isolated keto group. Generally, 3-hydroxyandrostane derivatives are more polar in adsorption HPLC with *n*-hexane–ethanol as eluent when the hydroxyl group is equatorial than when it is axial. 17β-Hydroxyandrostane derivatives are generally more polar than their 17α-epimers in reversed-phase partition HPLC with methanol–water as eluent. Compounds inseparable by adsorption HPLC can often be separated by reversed-phase partition HPLC and *vice versa*.

INTRODUCTION

In earlier work on the metabolism of labeled 4-androstene-3,17-dione in pea plants[1], we have felt the need for a fractionation method more efficient than thin-layer chromatography. Since then, we have studied the progesterone metabolites in pea plants[2] by high-performance liquid chromatography (HPLC)[3], which allowed us to isolate eleven labeled products. At present, we are studying the metabolism of androstenedione in male and female cucumber plants. Although we have previously devised a HPLC method for eleven androstane derivatives[4] and the literature contains additional methodology[5], we decided to make a systematic study of the relative merits of adsorption and reversed-phase HPLC of 69 androgen metabolites we may possibly encounter. It includes oxidation ($C_{19}O_3$) as well as reduction products of androstenedione. Most of them can be detected at 205 nm, but since some of the saturated alcohols cannot easily be detected that way, we have made extensive use of a refractive index (RI) detector.

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EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. The adsorption column was a 250 × 4.6 mm I.D. stainless-steel chromatography tube (Altex, Berkeley, CA, U.S.A.), packed with Zorbax BP-SIL (7–8 μm; DuPont, Wilmington, DE, U.S.A.). The reversed-phase column had the same dimensions, but it was packed with Zorbax BP-ODS (7–8 μm, DuPont). The columns were packed in our laboratory. The packing method, detectors, solvents, pump, and sample injection-valve were as previously described[3,6]. The chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

Our results are summarized in Table I. Sixty-nine androstane derivatives are arranged in the order of increasing oxygen content and increasing polarity in adsorption HPLC. The total number of hydroxyl and keto groups plays the most important role in the polarity of the androstane derivatives in both adsorption and reversed-phase partition HPLC. However, testosterone (30), the most polar $C_{19}O_2$ compound in adsorption HPLC, was somewhat more polar than 3α,11β-dihydroxy-5α-androstan-17-one (31), the least polar $C_{19}O_3$ compound. 4-Androstene-3β,17β-diol (23), the most polar $C_{19}O_2$ compound in reversed-phase partition HPLC, was well separated from the least polar $C_{19}O_3$ compound, 5β-androstane-3α,16α,17β-triol (60). Apparently, the effect of the total number of hydroxyl and keto groups on the polarity of androstane derivatives is more important in reversed-phase partition than in adsorption HPLC. Our previous results on triterpenoids[6], estrogens[7], and gibberellins[8] have likewise shown that the number of hydroxyl groups is the most important factor in the separation, but none of these compounds contained more than one keto group.

In adsorption HPLC (Fig. 1), 5β-androstane-3,17-dione (5) was eluted before 5α-androstane-3,17-dione (7) and 17α-hydroxy-4-androsten-3-one (27) was eluted before 17β-hydroxy-4-androsten-3-one (30). This is in agreement with most observations on the chromatographic behavior of epimeric steroids[9], but contrary to our previous results with dichloromethane–acetonitrile–2-propanol (179:20:1) as the eluent and Partisil 5 as the adsorbent[4]. When this eluent was used with the Zorbax BP-SIL column, the following retention times were observed: 5α-androstane-3,17-dione (7) (2.75 min), 5β-androstane-3,17-dione (5) (3 min), 17β-hydroxy-4-androsten-3-one (30) (8 min), 17α-hydroxy-4-androsten-3-one (27) (9 min). Thus, the sequences were the same as in our previous report[4], but the separations were not as good. The different order may be ascribed to the difference in eluents. In the adsorption systems 1 and 2 (Table I, Fig. 1) the 3-keto-5α-androstane derivatives (7, 10, 40) are more polar than their 5β-epimers (5, 8, 36), except 17β-hydroxy-5α-androstan-3-one (13) which is inseparable from its 5β-epimer (14). Thus, generally, the planar (A/B-*trans*) 3-ketosteroids are more strongly adsorbed than the folded (A/B-*cis*) 3-ketosteroids.

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

TABLE I

RETENTION TIMES OF ANDROSTANE DERIVATIVES

Hydroxyl groups are indicated by α and β, depending on orientation, at the position listed. However, at C-5, α and β are used to designate the orientation of hydrogen. Keto groups are indicated by O and double bonds by Δ. Hydroxyl groups at C-18 and C-19 are indicated by OH. Systems: 1, see Fig. 1; 2, see Fig. 2; 3, see Fig. 3; 4, see Fig. 4; 5, see Fig. 3, except that methanol–water (17:3) was used as eluent.

No.	*Substituents*									*Retention time (min)*				
										Systems				
	3	*4*	*5*	*6*	*11*	*16*	*17*	*18*	*19*	*1*	*2*	*3*	*4*	*5*
$C_{19}O$														
1	O	—	α	—	—	Δ	—	—	—	2	—	—	—	20
2	α	—	α	—	—	Δ	—	—	—	2.75	—	>60	—	18.25
3	β	—	Δ	—	—	—	—	—	—	4	—	—	—	23.5
4	β	—	α	—	—	Δ	—	—	—	4	—	—	—	20
$C_{19}O_2$														
5	O	—	β	—	—	—	O	—	—	7.5	—	15.5	—	—
6	O	—	Δ	—	—	—	O	—	—	8	—	17.5	—	—
7	O	—	α	—	—	—	O	—	—	8	—	17.75	—	—
8	O	—	β	—	—	—	α	—	—	8.5	—	23	—	—
9	β	—	β	—	—	—	O	—	—	10	—	14.25	—	—
10	O	—	α	—	—	—	α	—	—	10.25	—	18	—	—
11	α	—	α	—	—	—	O	—	—	12.5	—	16.25	—	—
12	β	—	Δ	—	—	—	O	—	—	12.5	—	11.25	—	—
13	O	—	α	—	—	—	β	—	—	12.5	—	16	—	—
14	O	—	β	—	—	—	β	—	—	12.5	—	15.75	—	—
15	β	—	α	—	—	—	O	—	—	13	—	14.75	—	—
16	α	—	β	—	—	—	O	—	—	13	—	16.25	—	—
17	β	—	β	—	—	—	β	—	—	16.5	—	11.75	—	—
18	α	—	α	—	—	—	β	—	—	18	—	16.75	—	—
19	β	—	β	—	—	—	α	—	—	19.25	—	16	—	—
20	O	Δ	—	—	—	—	O	—	—	20	—	11.5	—	—
21	β	—	Δ	—	—	—	β	—	—	20	—	10.25	—	—
22	β	—	α	—	—	—	β	—	—	20	—	13.5	—	—
23	β	Δ	—	—	—	—	β	—	—	20.25	—	9.75	41.5	—
24	α		α	—	—	—	α	—	—	20.25	—	33	—	—
25	β	—	Δ	—	—	—	α	—	—	20.5	—	11.25	—	—
26	β	—	α	—	—	—	α	—	—	20.5	—	14.5	—	—
27	O	Δ	—	—	—	—	α	—	—	21.5	7.5	12	—	—
28	α	—	β	—	—	—	α	—	—	24	7.0	32	—	—
29	α	—	β	—	—	—	β	—	—	24	7.5	15.5	—	—
30	O	Δ	—	—	—	—	β	—	—	25.75	8.75	10.75	—	—
$C_{19}O_3$														
31	α	—	α	—	β	—	O	—	—	—	8.5	—	19.75	—
32	α	—	β	—	β	—	O	—	—	—	9.25	—	17.5	—
33	β	—	Δ	—	β	—	O	—	—	—	9.25	—	13.25	—
34	O	Δ	—	—	β	—	O	—	—	—	9.5	—	13.75	—
35	β	—	Δ	—	—	β	O	—	—	—	9.5	—	13	—

(Continued on p. 218)

TABLE I *(continued)*

No.	*Substituents*									*Retention time (min)*				
	3	*4*	*5*	*6*	*11*	*16*	*17*	*18*	*19*	*Systems*				
										1	*2*	*3*	*4*	*5*
36	O	—	β	—	O	—	O	—	—	—	10	—	17.75	—
37	β	—	α	—	β	—	O	—	—	—	10	—	16.25	—
38	β	—	Δ	—	—	α	α	—	—	—	11.5	—	20.25	—
39	α	—	β	—	O	—	O	—	—	—	11.5	—	19.75	—
40	O	—	α	—	O	—	O	—	—	—	11.5	—	16.75	—
41	β	—	α	—	—	α	α	—	—	—	11.5	—	27.5	—
42	β	—	Δ	—	—	α	O	—	—	—	11	—	11.75	—
43	β	—	Δ	—	—	O	β	—	—	—	11.5	—	10	—
44	β	—	Δ	—	—	β	β	—	—	—	12	—	16.25	—
45	β	—	Δ	—	O	—	O	—	—	—	12.75	—	11.25	—
46	O	Δ	—	β	—	—	O	—	—	—	14.25	—	10.25	—
47	α	—	α	—	α	—	O	—	—	—	14.75	—	20.75	—
48	β	—	α	—	O	—	O	—	—	—	15.75	—	12.5	—
49	β	—	β	—	—	α	β	—	—	—	17.25	—	11.75	—
50	β	—	Δ	—	—	—	O	OH	—	—	17.5	—	9	—
51	O	Δ	—	—	β	—	β	—	—	—	17.5	—	15.5	—
52	α	—	α	—	β	—	β	—	—	—	18.25	—	18.5	—
53	β	—	Δ	—	—	α	β	—	—	—	18.75	—	8	—
54	β	—	Δ	—	β	—	β	—	—	—	19	—	13.75	—
55	α	—	β	—	O	—	β	—	—	—	19.25	—	22.75	—
56	β	—	α	—	—	α	β	—	—	—	19.5	—	11	—
57	β	—	α	—	β	—	β	—	—	—	20.25	—	17.5	—
58	α	—	β	—	β	—	β	—	—	—	20.5	—	17.25	—
59	β	—	Δ	—	—	—	O	—	OH	—	20.75	—	10.25	—
60	α	—	β	—	—	α	β	—	—	—	21	—	32	—
61	O	Δ	—	—	O	—	O	—	—	—	21.25	—	12.25	—
62	O	Δ	—	—	O	—	β	—	—	—	21.25	—	11.75	—
63	O	Δ	—	—	—	—	β	OH	—	—	21.5	—	19.75	—
64	O	Δ	—	—	—	α	O	—	—	—	23.5	—	11	—
65	O	Δ	—	—	—	—	O	—	OH	—	24.5	—	8	—
66	O	Δ	—	—	—	α	β	—	—	—	25.25	—	9	—
67	α	—	β	—	α	—	β	—	—	—	26.25	—	27.5	—
68	O	Δ	—	—	α	—	α	—	—	—	30	—	10.5	—
69	O	Δ	—	—	α	—	β	—	—	—	30	—	10.75	—

As shown in Table I, the 3-keto-4-androstene derivatives (20, 37, 30) were much more polar than their 5α-,5β-, and Δ^5-analogues (5, 6, 7; 8, 10; 13, 14) in both adsorption and reversed-phase partition HPLC. Steroids containing conjugated carbonyl groups are, as a rule, more polar than steroids with isolated keto groups. Their polarity was found to be comparable to the corresponding hydroxy steroids in both HPLC systems. In reversed-phase partition HPLC, the 3-keto-4-androstene derivatives (20, 27, 30, 34, 51, 61, 62, 66) were more polar than the corresponding 3-hydroxyandrostane derivatives (9, 11, 15, 16; 19, 24, 26, 28; 17, 18, 22, 29; 31, 32, 37; 52, 57, 58; 39, 48; 55; 49, 56, 60) but less polar than the 3-hydroxy-4 (or 5)-androstene derivatives (12; 25; 21, 23; 33; 54; 45; -; 53) (Table I and Fig. 3). We were unable to

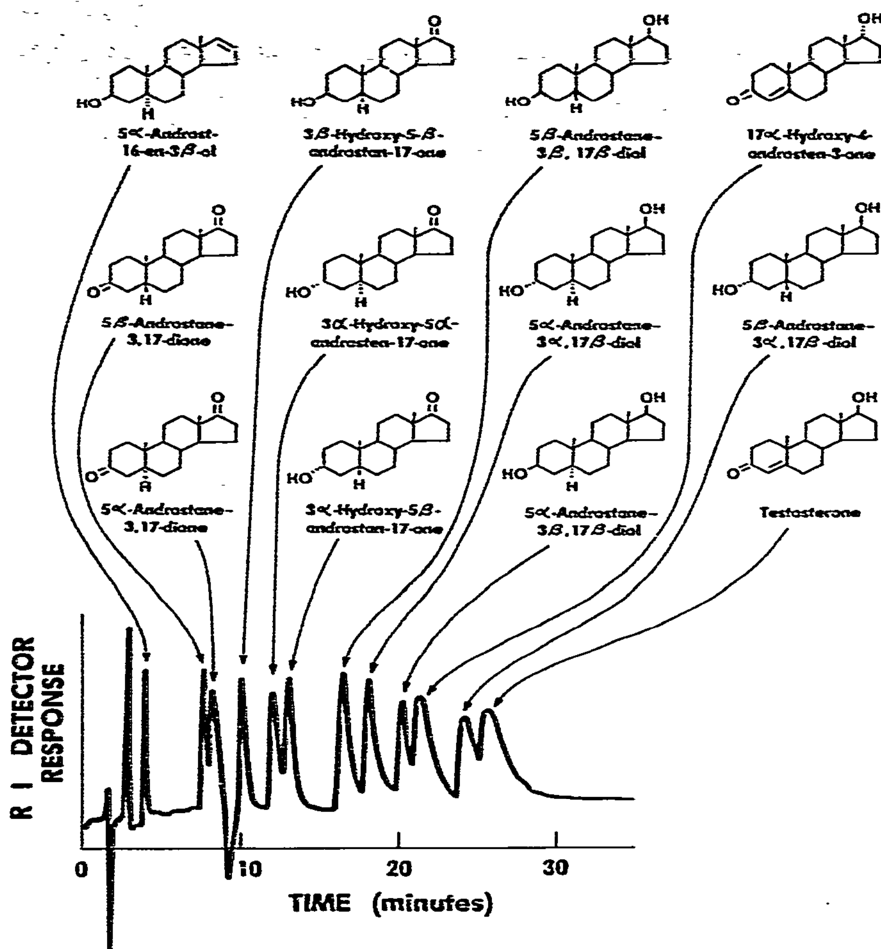

Fig. 1. Adsorption chromatogram of $C_{19}O$ and $C_{19}O_2$ androstane derivatives. Between 50 μg (5α-androst-16-en-3β-ol) and 300 μg (testosterone) of androstane derivatives, dissolved in 250 μl of the eluent, were chromatographed on a column of Zorbax BP-SIL, 250 × 4.6 mm I.D. Eluent *n*-hexane–ethanol (97:3); flow-rate, 2 ml/min; pressure, 600 p.s.i.; RI detector, 16×; recorder, speed 12 cm/h, span 10 mV.

separate the 3-hydroxy-4-androstene derivative, 4-androstene-3β,17β-diol (23) from its Δ^5- and 5α-analogues (21, 22), but separated it from its 5β-analogue (17) by adsorption HPLC. However, in reversed-phase partition HPLC, the Δ^4-, Δ^5-, 5α-, and 5β-analogues were readily separated (Table I and Fig. 4).

Table II summarizes the behavior of 3- and 5-epimers of 3-hydroxyandrostane derivatives in our adsorption (Figs. 1 and 2) and reversed-phase (Figs. 3 and 4) systems. Comparison of the effects of axial (a) and equatorial (e) hydroxyl groups (30 examples) showed that in adsorption HPLC steroids with equatorial 3-hydroxyl groups were consistently more polar than their axial epimers, except for 3β-hydroxy-5-androsten-17-one (12), which was inseparable from 3α-hydroxy-5α-androstan-17-one (11). This rule was also followed in reversed-phase partition HPLC, but several

TABLE II

RELATIVE POLARITIES OF 3- AND 5-ANALOGUES OF 3-HYDROXYANDROSTANE DERIVATIVES

> indicates that the first group of steroids is, as a rule, more polar than the second. A = adsorption, R = reversed-phase partition, + indicates that the rule shown in each heading is obeyed, − that it is violated, = indicates that analogues are inseparable. The superior HPLC system for each group of analogue separations is underlined.

Comparison between 3-OH(e) and 3-OH(a)

$3\alpha(e),5\beta > 3\alpha(a),5\alpha$		$\underline{A}$	R	$3\beta(e),5\alpha > 3\alpha(a),5\alpha$		A	$\underline{R}$
16	11	+	=	4	2	+	−
28	24	+	+	15	11	+	+
29	18	+	+	22	18	+	+
31	32	+	+	26	24	+	+
58	52	+	+	37	31	+	+
				57	52	+	+

$3\beta(e),5\alpha > 3\beta(a),5\beta$		$\underline{A}$	R	$3\alpha(e),5\beta > 3\beta(a),5\beta$		A	$\underline{R}$
9	15	+	−	16	9	+	−
22	17	+	−	28	19	+	−
26	19	+	+	29	17	+	−
56	49	+	+	60	49	+	−

$3\beta(e),\Delta^5 > 3\alpha(a),5\alpha$		A	$\underline{R}$	$3\beta(e),\Delta^5 > 3\beta(a)5\beta$		A	$\underline{R}$
12	11	=	+	12	9	+	+
21	18	+	+	21	17	+	+
25	24	+	+	25	19	+	+
33	31	+	+	53	49	+	+
54	52	+	+				

$3\beta(e),\Delta^4 > 3\alpha(a),5\alpha$		A	$\underline{R}$	$3\beta\ (e),\Delta^4 > 3\beta(a),5\beta$		A	$\underline{R}$
23	18	+	+	23	17	+	+

Comparison between 3-OH(e) and 3-OH(e)

$3\beta(e),5\alpha > 3\alpha(e),5\beta$		A	$\underline{R}$	$3\beta(e),\Delta^5 > 3\alpha(e),5\beta$		A	$\underline{R}$
15	16	=	+	12	16	−	+
22	29	−	+	21	29	−	+
26	28	−	+	25	28	−	+
37	32	+	+	33	32	=	+
48	39	+	+	53	60	−	+
56	60	−	+	54	58	−	+
57	58	−	−				

$3\beta(e),\Delta^4 > 3\beta(e),5\alpha$		A	$\underline{R}$	$3\beta(e),\Delta^4 > 3\beta(e),\Delta^5$		A	$\underline{R}$
23	22	+	+	23	21	+	+

$3\alpha(e),5\beta > 3\beta(e),\Delta^4$		A	$\underline{R}$
29	23	+	=

TABLE II (*continued*)

Comparison between 3-OH(a) and 3-OH(a)							
$3\alpha(a),5\alpha > 3\beta(a),5\beta$		*A*	*R*	$3\beta(a),\Delta^5 > 3\beta(a),5\alpha$		*A*	*R*
11	9	+	−	12	15	−	+
18	17	+	−	21	22	=	+
24	19	+	−	25	26	=	+
				33	37	−	+
				38	41	=	+
				45	48	−	+
				53	56	−	+
				54	57	−	+

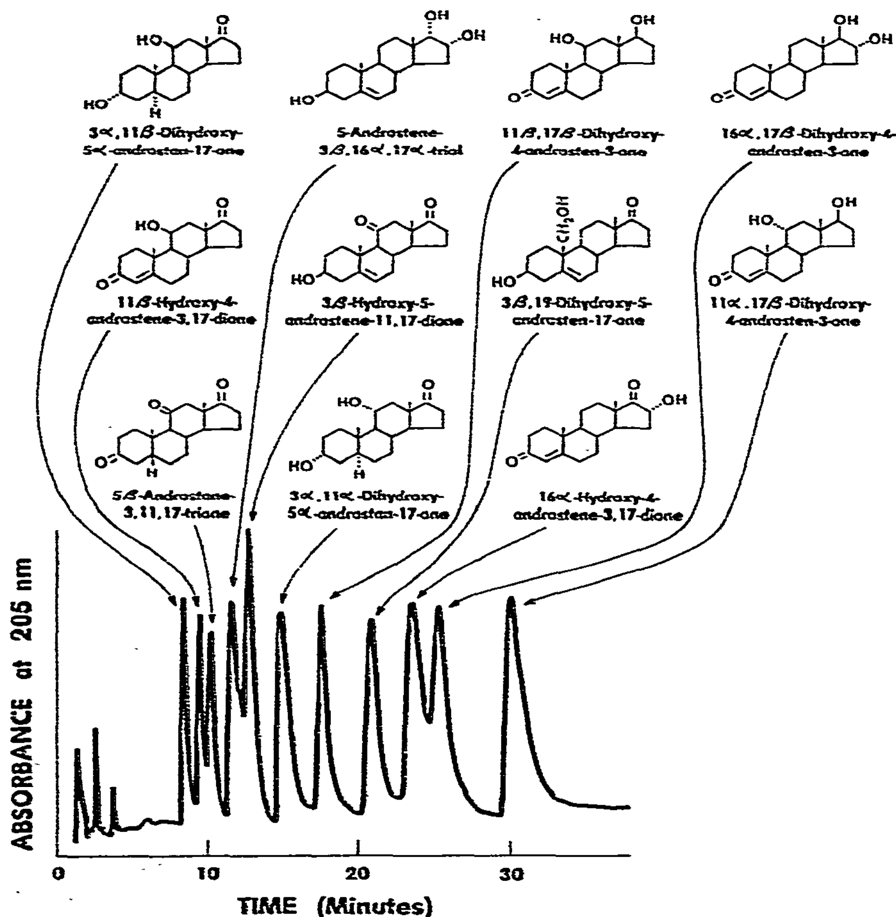

Fig. 2. Adsorption chromatogram of $C_{19}O_3$ androstane derivatives. Conditions as in Fig. 1, except between 5 μg (11β-hydroxy-4-androstene-3,17-dione) and 100 μg (3α,11α-dihydroxy-5α-androstan-17-one) of androstane derivatives, an eluent of *n*-hexane–ethanol (93:7), and a UV detector at 205 nm, range 0.2, time constant 1.0, were used.

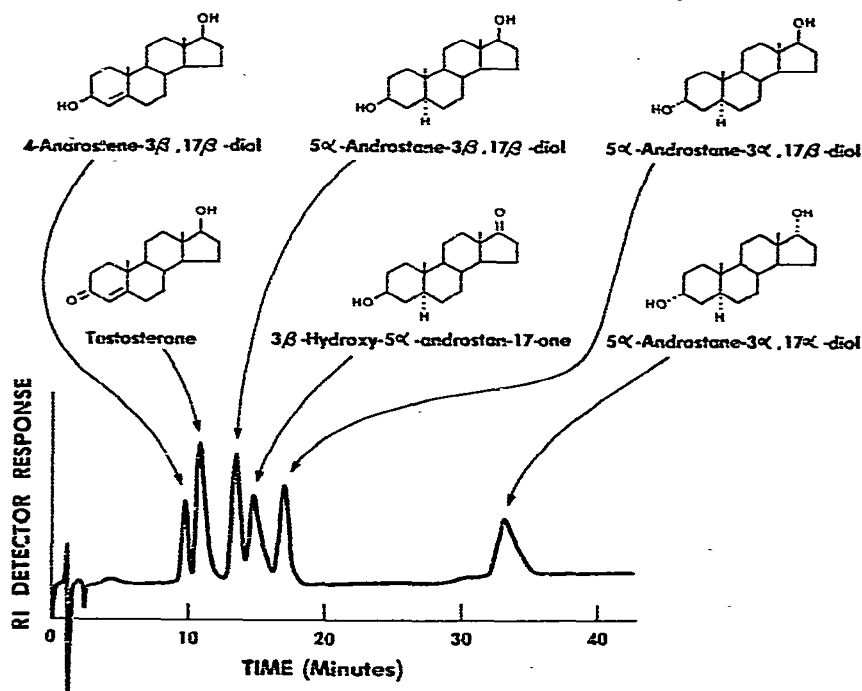

Fig. 3. Reversed-phase partition chromatogram of $C_{19}O_2$ androstane derivatives. About 100 μg of each androstane derivative, dissolved in 250 μl of the eluent, were chromatographed on a column of Zorbax BP-ODS, 250 × 4.6 mm I.D. Eluent, methanol–water (7:3); pressure, 100 p.s.i.; RI detector, 8×. Other conditions as in Fig. 1.

exceptions were noted. We have observed that adsorption HPLC separates 5-epimers better than reversed-phase partition HPLC, while reversed-phase partition HPLC separates 3-epimers better than adsorption HPLC.

Comparison of 5-analogues with equatorial 3-hydroxyl groups (16 examples) showed that, generally, the 3β-hydroxy-5α-androstane derivatives and the 3β-hydroxy-5-androstene derivatives are more polar than the 3α-hydroxy-5β-androstane derivatives in reversed-phase partition HPLC, while in adsorption HPLC the 3α-hydroxy-5β-androstane derivatives are more polar than the 3β-hydroxy-5-androstene derivatives. For this group of steroids, reversed-phase partition HPLC was found to give separations superior to those by adsorption HPLC.

Comparisons of 5-analogues with axial 3-hydroxyl groups (11 examples) showed that, generally, the 3α-hydroxy-5α-androstane derivatives were more polar than the 3β-hydroxy-5β-androstane derivatives in adsorption HPLC, while the 3β-hydroxy-5β-androstane derivatives were more polar than the 3α-hydroxy-5α-androstane derivatives in reversed-phase partition HPLC. The 3β-hydroxy-5-androstene derivatives were more polar than the 3β-hydroxy-5α-androstane derivatives in reversed-phase partition HPLC, but in adsorption HPLC the 3β-hydroxy-5α-androstane derivatives were either more polar than the 3β-hydroxy-5-androstene derivatives or inseparable from them. For this group of steroids reversed-phase partition HPLC was also found to provide greater resolving power.

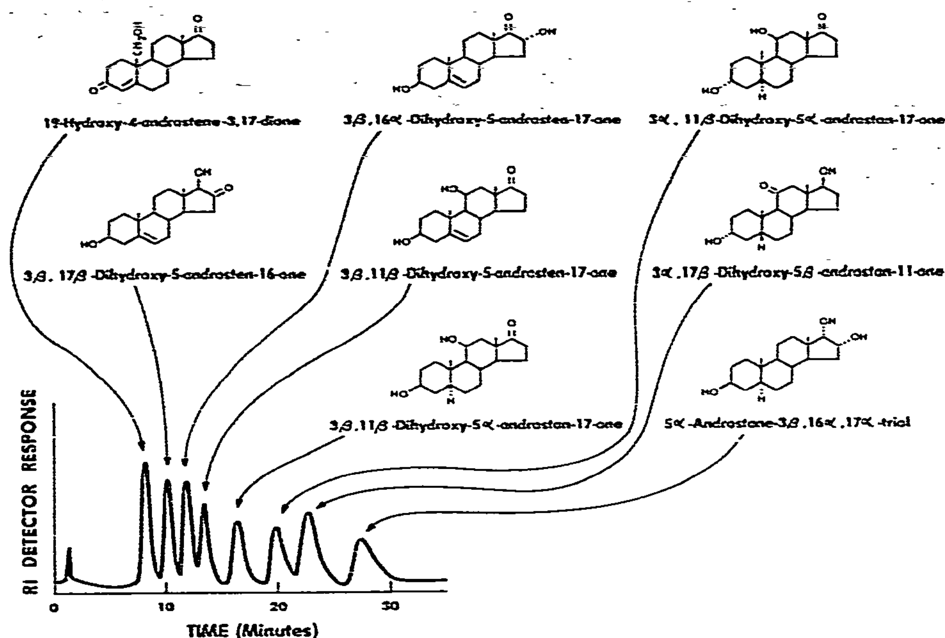

Fig. 4. Reversed-phase partition chromatogram of $C_{19}O_3$ androstane derivatives. Conditions as in Fig. 3 except an eluent of methanol–water (11:9) was used and the pressure was 1100 p.s.i.

All of the 17β-hydroxyandrostane derivatives in Table I (13, 14, 17, 18, 21, 22, 29, 30, 53, 56, 59) were more polar than their 17α-epimers (10, 8, 19, 24, 25, 26, 28, 27, 38, 41, 68) in reversed-phase partition HPLC. 11β-Hydroxyandrostane derivatives (31, 51, 58) were less polar than their 11α-epimers (47, 69, 67) in adsorption HPLC. Adsorption HPLC separated the 11-epimers better than reversed-phase partition HPLC. The 16β-hydroxyandrostane derivatives (35, 44) were less polar than their 16α-epimers (42, 53) in both HPLC systems.

In general, hydroxyl groups contribute more to the polarity of the steroids than keto groups, but an α,β-unsaturated keto group makes them about as polar as a hydroxyl group. The number of hydroxyl groups and keto groups, the locations of both groups and of double bonds, the configuration at all chiral centers, the sorbent, and the eluent determine the elution order of androstane derivatives in HPLC. Adsorption and reversed-phase partition HPLC complement each other, and most androstane derivatives can be separated by a combination of both methods.

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