Journal of Chromatography, 287 (1984) 105–112
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,482

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF STEROIDAL SAPOGENINS

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(First received October 10th, 1983; revised manuscript received December 5th, 1983)

SUMMARY

Thirty steroidal sapogenins were chromatographed by normal-phase and reversed-phase high-performance liquid chromatography (HPLC). These two HPLC systems complement each other and as result most sapogenins can be separated. The total number of hydroxyl and keto groups plays the most important role in the polarity of spirostane derivatives in both normal-phase and reversed-phase HPLC. Generally, 3-hydroxysapogenins are more polar in the normal-phase HPLC with hexane–ethanol as eluent when the hydroxyl group is equatorial than when it is axial. 25-Epimers of sapogenins cannot be separated well by either system.

INTRODUCTION

The steroidal sapogenins are a group of widely distributed plant products useful for the manufacture of pharmaceutically important steroids. High-performance liquid chromatography (HPLC) of very few steroidal sapogenins has been reported. The benzyl esters of hecogenin, $\Delta^{9(11)}$-dehydrohecogenin, tigogenin, sarsasapogenin, $\Delta^{9(11)}$-dehydrotigogenin and diosgenin have been chromatographed by a reversed-phase HPLC[1]. The acetates of diosgenin, yamogenin, tigogenin, neotigogenin, smilagenin and sarsasapogenin have been separated by the combination of both normal-phase and reversed-phase HPLC[2]. These derivatives were detected by a UV detector. Reports on the HPLC of underivatized free steroidal sapogenins using an RI detector include the normal-phase HPLC determination of diosgenin in plants[3], reversed-phase HPLC separation of hecogenin, tigogenin and diosgenin[4] and the reversed-phase HPLC estimation of the amounts of hecogenin and tigogenin in plants[5]. In this communication we report both the normal-phase and reversed-phase HPLC of 30 underivatized free steroidal sapogenins. These HPLC systems can be used in the metabolic studies as we previously reported in steroid hormone metabolism[6] and also can be used in the analysis of sapogenins in plants.

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TABLE I

RETENTION TIMES OF STEROIDAL SAPOGENINS

Hydroxyl groups are indicated under OH column 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 under O column and double bonds under Δ column or under C-5 column. Methyl groups at C-25 are indicated under C-25 column by *R* and *S*, depending on orientation. For complete structures, see Figs. 1–5. Systems: 1, see Fig. 1; 2, see Fig. 2; 3, see Fig. 3; 4, see Fig. 4. Capacity factors, $k' = (t - t_0)/t_0$, t_0 = 1.25 min (normal-phase, systems 1 and 2), t_0 = 0.90 (reversed-phase, system 3), t_0 = 1.20 min (reversed-phase, system 4).

No.	*Compound*	*Structure*					*Retention time (min)*			
		OH	*O*	*Δ*	*C-5*	*C-25*	*Systems*			
							1	*2*	*3*	*4*
1	Diosgenone		3	4		*R*	3.75		15.25	
2	Smilagenin	3β			β	*R*	3.75		22.25	
3	Sarsasapogenin	3β			β	*S*	4		22.5	
4	6-Methyldiosgenin	3β			Δ	*R*	4.25		26	
5	3-Episarsasapogenin	3α			β	*S*	4.5		21	
6	Yamogenin	3β			Δ	*S*	4.5		21.75	
7	Diosgenin	3β			Δ	*R*	4.5		21	
8	Tigogenin	3β			α	*R*	4.75		24.5	
9	Neotigogenin	3β			α	*S*	4.75		25	
10	Bethogenin	3β			Δ	*R*	6.75		12.25	
11	5,6-Dihydrobethogenin	3β			α	*R*	6.75		13.5	
12	Pennogenin	3β,17α			Δ	*R*	7.25		7	
13	Hecogenin	3β	12		α	*R*	12.75	4.25	6.0	32.75
14	Gentrogenin	3β	12		Δ	*R*	13.75		5.5	29
15	Pseudosmilagenin	3β,27		20(22)	β	*R*	17	4.5	4.75	20
16	5α-Hydroxy-6β-methyltigogenin	3β,5α				*R*	20	4.75	9.5	
17	(25*R*)-5α-Spirostan-3β,12β-diol-11-one	3β,12β	11		α	*R*	21	5	4.5	20
18	Pseudosarsasapogenin	3β,27		20(22)	β	*S*	21	5	6	
19	11-Ketotigogenin	3β	11		α	*R*	22	5.5	5.75	30.5
20	Gitogenin	2β,3β			α	*R*	26.5	5.5	4.5	23
21	Pseudokryptogenin	3β,27		16,20(22)	Δ	*R*		6	6.5	
22	3-Epirockogenin	3α,12β			α	*R*		6.5		19
23	Rockogenin	3β,12β			α	*R*		7		21.5
24	(25*S*)-5α-Spirostan-3β,12β-diol	3β,12β			α	*S*		7		22.25
25	11α-Hydroxytigogenin	3β,11α			α	*R*		7.5		24
26	Pseudodiosgenin	3β,27		20(22)	Δ	*R*		8	5.75	37.5
27	16-Deoxokryptogenin	3β,27	22		Δ	*R*		8.75		21.5
28	11α-Hydroxy-7-ketodiosgenin	3β,11α	7		Δ	*R*		15.75		7.75
29	Kryptogenin	3β,27	16,22		Δ	*R*		17		7

EXPERIMENTAL

The HPLC system consisted of a pump (Model 110A, Altex, Berkeley, CA, U.S.A.) with a high sensitivity pressure filter (Altex), a sample injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.), an RI detector (Model R401, Waters, Milford, MA, U.S.A.) and a recorder (Model 385, Linear, Irvine, CA, U.S.A.). A variable wavelength UV-VIS detector (Model 155-30, Altex) at 205 nm was also used instead of an RI detector when steroidal sapogenins containing a double bond at C-4 or 5 position were analyzed. The prepacked normal-phase column (Spherisorb S-5-W, silica, 5 μm, 25 × 0.46 cm I.D., Alltech, Deerfield, IL, U.S.A.) and the prepacked reversed-phase column (Ultrasphere ODS, 5 μm, 25 × 0.46 cm I.D., Altex) were used. The chromatographic conditions are given in figure legends.

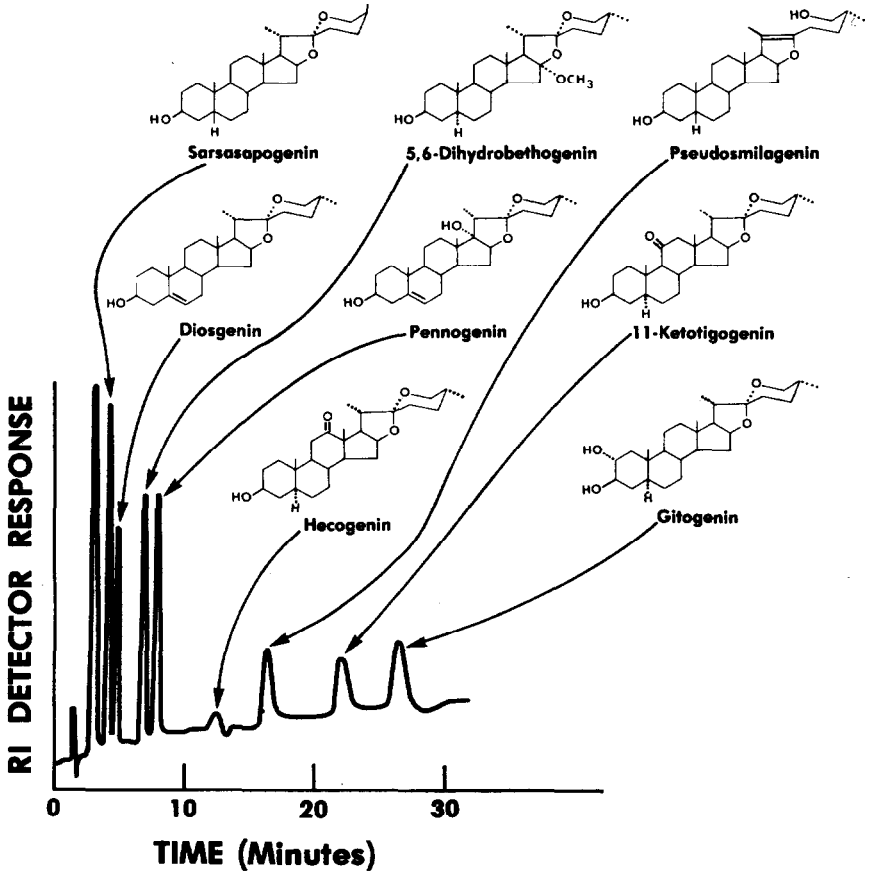

Fig. 1. Normal-phase chromatogram of less polar steroidal sapogenins. Between 50 μg (sarsasapogenin) and 200 μg (gitogenin) of sapogenins, dissolved in 200 μl of the eluent (very small amount of ethanol was added to help to dissolve sapogenins) were chromatographed on a column of Spherisorb S-5-W, 25 × 0.46 cm I.D. Eluent, hexane–ethanol (98:2); flow-rate, 2 ml/min; pressure, 1000 p.s.i.; RI detector, 10×; recorder speed 12 cm/h, span 10 mV.

RESULTS AND DISCUSSION

The results are summarized in Table I. Thirty steroidal sapogenins are arranged in the order of increasing polarity in the normal-phase HPLC. The total number of hydroxyl and keto groups plays the most important role in the polarity of spirostane derivatives in both normal-phase and reversed-phase HPLC. An additional hydroxyl or a keto group significantly increases the polarity of the molecule in both normal-phase and reversed-phase HPLC systems. In Table I the total number of hydroxyl and keto groups is one for compounds 1–11, two for compounds 12–26, and three for compounds 27–30, except Nos. 17 (three) and 29 (four). This HPLC behavior parallels to that of androgens[7] and estrogens[8].

We have reported both the normal-phase and reversed-phase HPLC of 69 androgens[7] and have concluded that 3-hydroxyandrostane derivatives are more polar in normal-phase HPLC with hexane–ethanol as eluent when the 3-hydroxyl group is equatorial than when it is axial. This present study of steroidal sapogenins as shown in Table II agrees with this conclusion. The HPLC of progestane derivatives[9] also

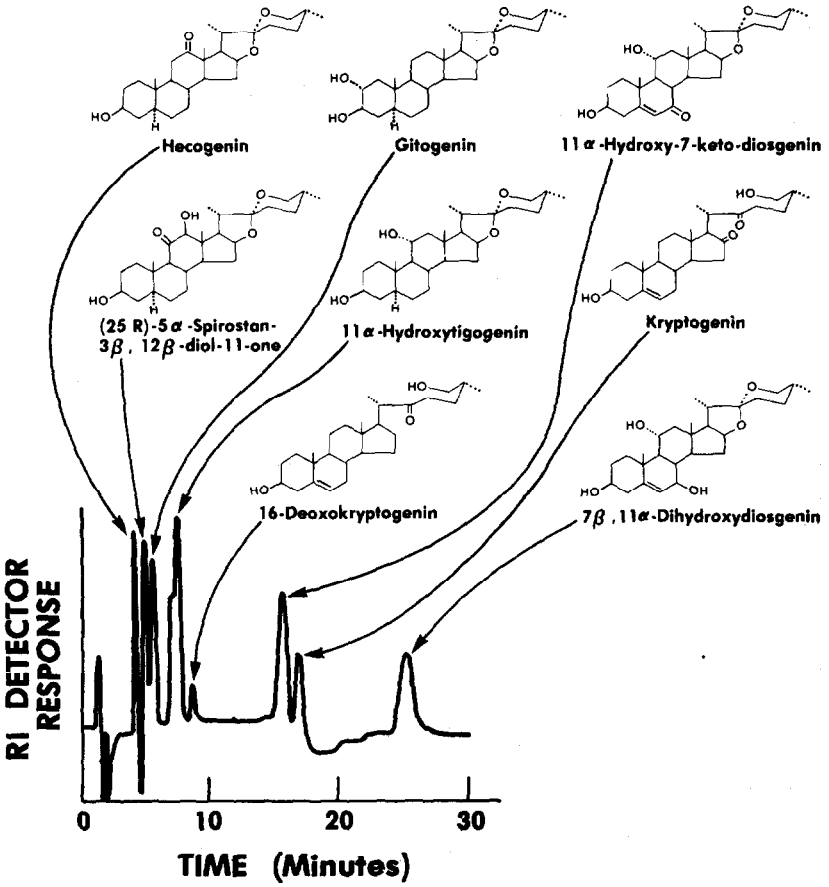

Fig. 2. Normal-phase chromatogram of more polar steroidal sapogenins. Conditions as in Fig. 1 except an eluent of hexane–ethanol (93.5:6.5) was used.

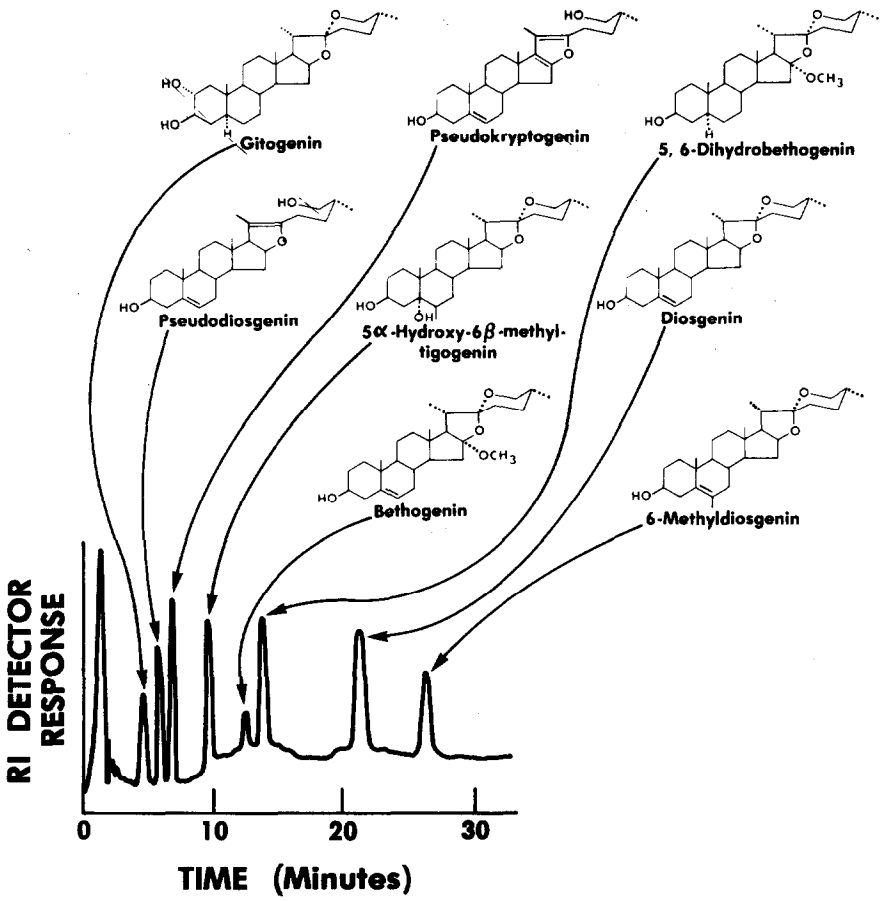

Fig. 3. Reversed-phase chromatogram of less polar steroidal sapogenins. Conditions as in Fig. 1 except a column of Ultrasphere ODS, 25 × 0.46 cm I.D. and an eluent of methanol–water (85:15) were used. Sapogenins were dissolved in 150 μl of methanol before injection. Pressure, 3500 p.s.i.

showed that the equatorial 3-hydroxyl group contributes to polarity of the molecule more than the axial 3-hydroxyl group in normal-phase HPLC with the eluents of dichloromethane–ethanol (99.75:0.25) or hexane–isopropanol (97:3). Comparisons between equatorial 3-hydroxyspirostane derivatives are also shown in Table II. These analogs are either inseparable or not separated well in the normal-phase HPLC (Table I). However, they can be separated using the reversed-phase HPLC (Table I).

A hydroxyl group in general contributes to the polarity of the molecule more than an isolated keto group (unconjugated) in both normal-phase and reversed-phase HPLC. For example, rockogenin (No. 23 in Table I) is more polar than hecogenin (No. 13) and 11α-hydroxytigogenin (No. 25) is more polar than 11-ketotigogenin (No. 19). However, an α,β-unsaturated keto group such as the keto group in diosgenone (No. 1) contributes as much to the polarity of the molecule as a hydroxyl group. A tertiary hydroxyl group at C-17 (No. 12) contributes to polarity of the molecule less than an isolated keto group (Nos. 13, 14 and 19) in both normal-phase

and reversed-phase HPLC. A tertiary hydroxyl group at C-5 (No. 16) contributes to polarity of the molecule either less than an isolated keto group (No. 19) or more than an isolated keto group (Nos. 13 and 14) in both normal-phase and reversed-phase HPLC.

The order of decreasing contribution of hydroxyl groups of 3β-hydroxyspirostane derivatives to the polarity of the molecule is 11α > 12β > 2β > 5α > 17α such as 11α-hydroxytigogenin (No. 25) > rockogenin (No. 23) > gitogenin (No. 20) > 5α-hydroxy-6β-methyltigogenin (No. 16) > pennogenin (No. 12). The order in the reversed-phase HPLC is 12β > 2β > 11α > 17α > 5α. The degree of the contribution of the hydroxyl group to polarity depends on the location of the hydroxyl group on the molecule and also on the HPLC system.

The methoxyl group at C-16 (Nos. 10 and 11) contributes to the polarity of the molecule less than hydroxyl or keto group but more than the methyl group at C-6 (No. 4) in both normal-phase and reversed-phase HPLC. A cyclic ether at ring F (Nos. 2, 3, 7) contributes to polarity of the molecules less than the hydroxyl group

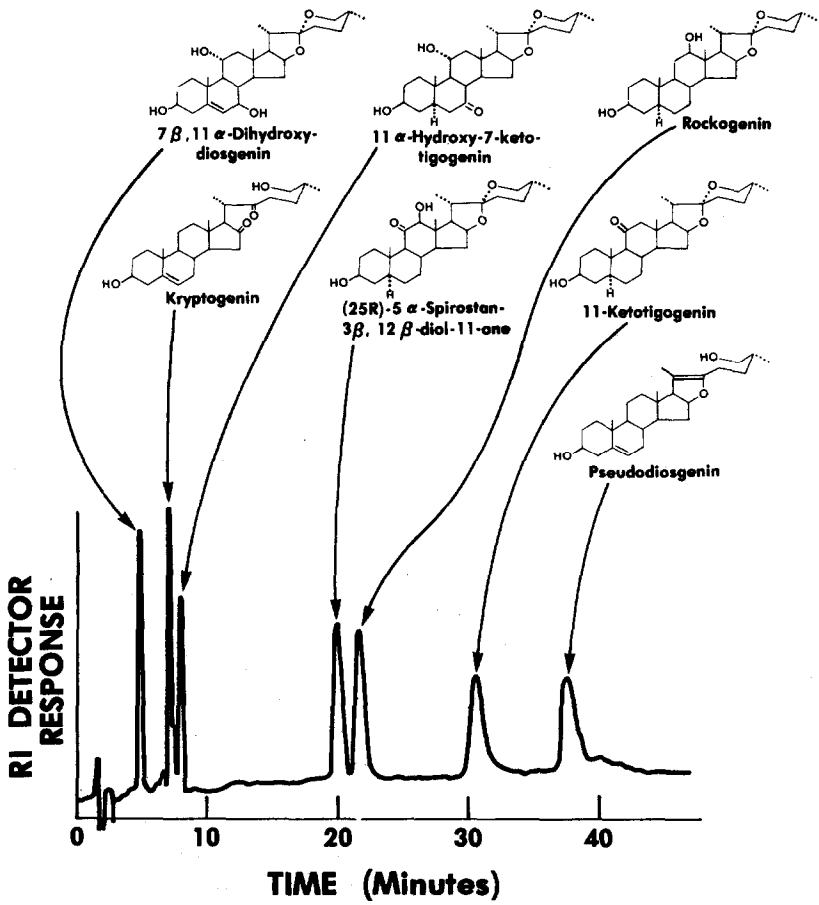

Fig. 4. Reversed-phase chromatogram of more polar steroidal sapogenins. Conditions as in Fig. 3 except an eluent of methanol–water (73:27) and flow-rate of 1.5 ml/min were used.

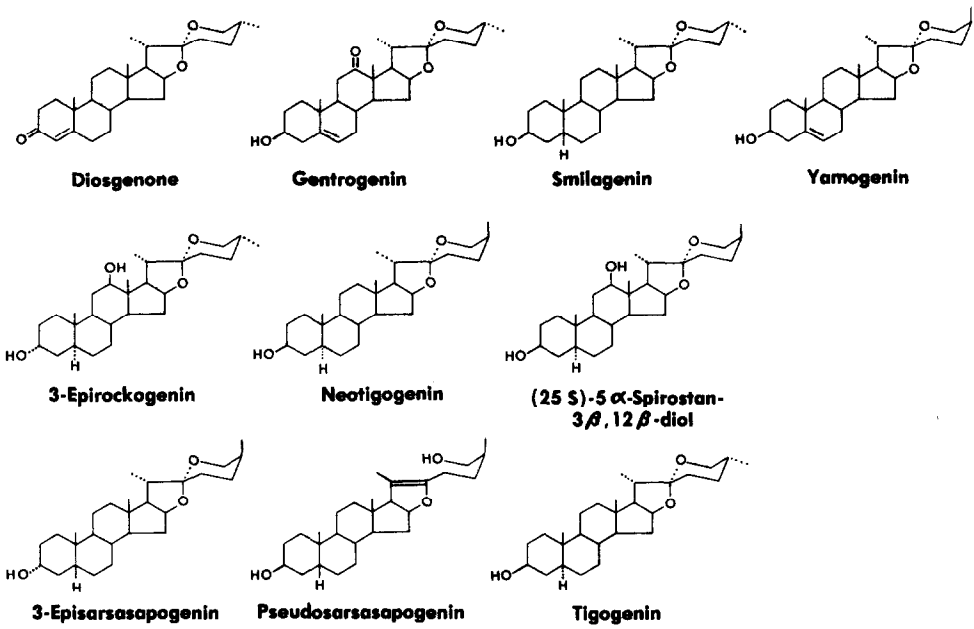

Fig. 5. Additional steroidal sapogenin structures.

at C-27 (Nos. 15, 18, 26) in both HPLC systems. Pseudokryptogenin (No. 21) with an additional double bond at C-16 is less polar than pseudodiosgenin (No. 26) in both normal-phase and reversed-phase HPLC systems.

25-Epimers of steroidal sapogenins as shown in Table I cannot be separated well with either normal-phase or reversed-phase HPLC. As shown in Table II, they are either completely inseparable or that 25*R*-sapogenins are less polar than their 25*S*-analogs in the normal-phase HPLC. 25*R*-sapogenins are more polar than their 25*S*-analogs in the reversed-phase HPLC.

Tal and Goldberg[4] used a C_{18} column with the eluent of acetonitrile–methanol–chloroform (83:10:7). The elution order which was different from that found in this study (Table I) was hecogenin, tigogenin and diosgenin. The difference is due to the fact that we use true reversed-phase partition systems, whereas the chromatographic behavior in water-free systems is probably governed by hydrophobic adsorption. Sitton *et al.*[5] used a C_{18} column with the eluent of acetonitrile–water–methanol–chloroform (73:20:6:1) and the elution order was rockogenin, hecogenin and tigogenin which was the same as that of our present study (Table I). Hunter *et al.*[2] have completely separated C-25 epimers in the form of their acetates, diosgenin acetate–yamogenin acetate and smilagenin acetate–sarsasapogenin acetate, using normal-phase HPLC. The underivatized C-25 epimers are poorly separated by both normal-phase and reversed-phase HPLC (Table I).

The normal-phase and reversed-phase HPLC of underivatized sapogenins (Table I) complement each other and most of the separations can be achieved. The compounds inseparable by both systems can be separated by the HPLC of their corresponding acetates. We have reported both the normal-phase and reversed-phase HPLC of free androgens[7] and their acetates[6]. These HPLC methods have been used for the identification of radioactive metabolites of 4-[4-^{14}C]androstene-3,17-dione in growing cucumber plants[6]. Ten radioactive metabolites were identified.

TABLE II

RELATIVE POLARITIES OF STEROIDAL SAPOGENINS

> Indicates that the first group of steroids is, as a rule, more polar than the second. N = normal-phase, R = reversed-phase, e = equatorial, a = axial, + indicates that the rule shown in each heading is obeyed, − indicates that it is violated, = indicates that analogues are inseparable. The superior HPLC system for each group of analogue separations is underlined. The compound numbers are the same as those in Table I.

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

3β(e),5α >	*3β(a),5β*	*N*	*R*	*3β(e),Δ⁵* >	*3β(a),5β*	*N*	*R*
8	2	+	−	6	3	+	+
9	3	+	−	7	2	+	+
				26	15	+	−
3α(e),5β >	*3β(a),5β*	*N*	*R*	*3β(e),5α* >	*3α(a),5α*	*N*	*R*
5	3	+	+	23	22	+	−

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

3β(e),5α >	*3β(e),Δ⁵*	*N*	*R*	*3α(e),5β* >	*3β(e),Δ⁵*	*N*	*R*
8	7	+	−	5	6	=	+
9	6	+	−				
11	10	=	−	*3α(e),5β* >	*3β(e),5α*	*N*	*R*
13	14	−	−	5	9	−	+

Comparison between 25R and 25S (methyl group orientation)

25R >	*25S*	*N*	*R*	*25R* >	*25S*	*N*	*R*
2	3	−	+	15	18	−	+
7	6	=	+	23	24	=	+
8	9	=	+				

ACKNOWLEDGEMENTS

Gifts of reference compounds from Dr. Erich Heftmann and the Medical Research Council (Dr. D. N. Kirk, Westfield College, Hampstead, London, U.K.) are gratefully acknowledged.

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