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129. Shoji Hara and Michiko Miyaki (née Takeuchi): Systematic Analysis of Steroids. VI.*1 Relationship between Steroid Structure and Mobility in Adsorption Liquid Phase Chromatography.*2

(*Division of Organic Chemistry, Tokyo College of Pharmacy**3)

The quantitative relationship between chromatographic mobility and steroid structure has been investigated. The group Rm increments were calculated from the mean Rf values of a number of steroid hormones, sapogenins, and bile acids.

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In order to apply liquid phase chromatography to structural determination of steroids, it is first necessary to clarify the quantitative relationship between the migration of a steroid and its structure. In the case of the liquid-liquid or gas-liquid partition system, some systematic chromatographic studies of steroids[1] have been reported previously on the basis of Martin's theory.[2] However, in the case of the liquid-solid adsorption chromatographic system which is widely used as a separation technique, quantitative treatment of the mobility has not been considered possible up to now.

Already, one of the authors (S. H.)[3~6] has carried out a systematic and simultaneous analysis of steroids by thin-layer chromatography utilizing silica gel as adsorbent, for identification of natural steroids. For an extension of these studies, empirical rules for the behavior of steroids in adsorption chromatography have been investigated in this paper. The Rm-function as defined by Bate-Smith and Westall,[7] and the ΔRm value, *i.e.*, the increment in the Rm value of a steroid when a substituent is introduced, were calculated from the mean Rf values of a number of steroid hormones, sapogenins and bile acids. It was found that structural change affords nearly characteristic parameters, if suitable developers have been selected.

Recently, Lisboa[8~10] and Fehér[11] also investigated the mobilities of a great number of C_{19}-[8~11] and C_{21}-steroids,[10] and calculated the ΔRm values by a similar method.

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*1 Part V: This Bulletin, **12**, 626 (1964).

*2 This work was presented at the Pharmaceutical Society of Japan in Tokyo, November 1963. A brief report of this paper appeared as a Communication to the Editor in J. Chem. Soc. Japan, **86**, 1344 (1964) (Japanese).

*3 Ueno-Sakuragi, Taito-ku, Tokyo (原 昭二, 宮木(旧姓竹内)美知子).

1) I. E. Bush: Chromatography of Steroids, Pergamon Press, Oxford, p. 83 (1961); R. Neher: Steroid Chromatography, Elsevier, Amsterdam, p. 228 (1964); D. M. Cathro, J. Cameron, K. Birchall: J. Chromatog., **17**, 362 (1965), etc.
2) A. J. P. Martin: Symp. Biochem. Soc., **3**, 4 (1949).
3) S. Hara, M. Takeuchi: Endocrinol. Japonica, **10**, 202 (1963).
4) M. Takeuchi: This Bulletin, **11**, 1183 (1963).
5) K. Takeda, S. Hara, A. Wada, N. Matsumoto: J. Chromatog., **11**, 562 (1963).
6) S. Hara, M. Takeuchi: J. Chromatog., **11**, 565 (1963).
7) E. C. Bate-Smith, R. G. Westall: Biochim. Biophys. Acta, **4**, 427 (1950).
8) B. P. Lisboa: J. Chromatog., **19**, 81 (1965).
9) *Idem*: *Ibid.*, **19**, 333 (1965).
10) *Idem*: Steroids, **6**, 605 (1965).
11) T. Fehér: J. Chromatog., **19**, 551 (1965).

Materials and Methods

As adsorbent, hydrophilic silica gel for thin-layer chromatography containing 5% gypsum (Wakogel B-5, Wako Pure Chemical Co., Tokyo) was used. To make a thin layer (250 μ in thickness), twice the volume of water was added, then 20×20 cm. glass plates were coated uniformly with the resulting suspension by a commercial spreader. The plates were allowed to dry for about 30 mins. in the atmosphere and activated at 110° for one hour.

Each steroid sample in 1% chloroform or methanol solution was applied to the thin layer. After development, spots were detected by spraying with conc. sulfuric acid and heating to 110° for 10 mins.

The experimental conditions required for reproducibility of Rf values as well as for close resemblance between "observed" Rf value[12] obtained in "plate chromatography"*4,[13] and "theoretical" or "true" Rf value,[12] are described as follows :

1) Development was carried out at a constant temperature (16±1°). 2) The quantity of sample applied to the thin layer was 2 to 3 μg. 3) The starting line was placed at 15 mm. from the lower edge of the plate, and the immersion line was at 10 mm. Distance between the solvent front and starting point was 13 cm. 4) The ascending development was run in a new "sandwich" type chamber[14] saturated by the solvent vapor. 5) As a rule only Rf values in a limited range between 0.1 to 0.7 were adopted, because the volume ratio of stationary phase to mobile phase is not constant at the front and at a little upper portion of the immersion line.[12] 6) For binary solvent systems, those which were not subjected to frontal analysis[12] were used. 7) For simultaneous analysis, 26 steroid hormones,[4] 20 steroid sapogenins,[5] and 57 bile acid derivatives[6] were spotted individually as well as in mixtures. Rf values were determined by the separation of each component from a mixture of respective group. 8) Only "observed" Rf values found within a range of 0.03 were selected. Mean Rf values were obtained from those "observed" values in five or more experiments. Rm-functions were calculated according to the following equation[7] : $Rm = \log(1/Rf - 1)$.

Results and Discussion

ΔRm values due to the change in the molecular structure were calculated from the mean Rf values of a pair of steroids having the common skeleton. It has been shown that the group parameters varied depending on the character of developing solvents, especially, if hydroxylic solvents such as methanol and ethanol were contained in the solvent system. These results suggest that the hydrogen bond formed between the siloxan structure in silica gel and the polar functional group of the sample was affected by the hydroxylic solvent. Alcohol-containing solvent systems such as chloroform–ethanol or methanol and benzene–ethanol or methanol have been widely used for separation of steroid mixtures by silica gel thin-layer chromatography. However, it was observed that these developers were not suitable for calculation of group parameters in the present study. From a number of solvents, the benzene–acetone system was selected on the basis of good reproducibility of Rf values and reliable constancy of calculated group parameters for steroid hormones and sapogenins. Similarly, suitable solvents were selected for bile acids and their derivatives. In Table I the mean Rf values of the root compounds and ΔRm values of respective functional groups are summarized.

Two exceptional values in this table (indicated by parentheses), are most likely due to reciprocal interaction by neighboring functional groups. However, it can be seen that the adsorptivity of hydroxyl or carbonyl groups in different positions or configurations may be expressed as the characteristic group parameters. ΔRm values

*4 A general term for chromatography which has a plate-type filling system of supports, as against a column-type filling system, *viz.*, thin-layer and paper chromatography.

12) M. Brenner, A. Niederwieser, G. Pataki, R. Weber in E. Stahl (Editor) : Dünnschicht-Chromatographie, Springer-Verlag, Berlin, p. 108 (1962).

13) S. Hara : Liquid Phase Chromatography of Organic Compounds, Kyoritsu Publ. Co., Tokyo, p. 29 (1965) (in Japanese); S. Hara in S. Hara, O. Tanaka, S. Takitani (Editors) : Thin-Layer Chromatography, Vol. I, Nankodo Publ. Co., Tokyo, p. 8 (1964) (in Japanese).

14) S. Hara, M. Takeuchi, N. Matsumoto : Japan Analyst, **13**, 359 (1964).

Table I. ΔRm Values of Converted Functional Groups for Steroids

Converted functional group	Root compound	"Observed" mean Rf value	ΔRm value	Solvent[a] system
Steroid hormone				
11β-OH	21-Hydroxy-pregn-4-ene-3,20-dione	0.56	+0.36	a
	17α,21-Dihydroxy-pregn-4-ene-3,20-dione	0.45	+0.31	a
16α-OH	Estra-1,3,5(10)-triene-3,17β-diol	0.58	+0.73	a
17α-OH	21-Hydroxy-pregn-4-ene-3,20-dione	0.56	+0.17	a
11=O	Androst-4-ene-3,17-dione	0.71	(+0.08)	b
	3α-Hydroxy-5α-androstan-17-one	0.61	+0.22	b
	17α,21-Dihydroxy-pregn-4-ene-3,20-dione	0.45	+0.18	a
3α-OH to 3β-OH	3α-Hydroxy-5α-androstan-17-one	0.61	+0.07	b
20β-OH to 20α-OH	5α-Pregnane-3β,20β-diol	0.56	+0.07	a
4,5α-H to Δ^4	5α-Pregnane-3,20-dione	0.78	+0.07	b
Steroid sapogenin				
1β-OH	Yonogenin (C_{25} : D, A/B : cis, 2β,3α-OH)	0.09	+0.63	c
2α-OH	Tigogenin (D, trans, 3β-OH)	0.52	+0.79	d
12=O	Tigogenin (D, trans, 3β-OH)	0.40	+0.35	c
	Diosgenin (D, Δ^5, 3β-OH)	0.41	+0.29	c
5α,6-H to Δ^5	Tigogenin (D, trans, 3β-OH)	0.40	−0.03	c
	Hecogenin (D, trans, 3β-OH, 12=O)	0.23	−0.08	c
C_{25}-D to L	Meteogenin (D, $\Delta^{1,3,5}$, 1-Me, 11α-OH)	0.68	+0.01	c
	Isorhodeasapogenin (D, cis, 1β,3β-OH)	0.22	±0.00	c
Methyl cholanate derivative				
3=O	Methyl 7-oxo-5β-cholanate	0.68	+0.76	e
7=O	Methyl 3-oxo-5β-cholanate	0.65	+0.70	e
	Methyl 3α-acetoxy-12-oxo-5β-cholanate	0.52	+0.54	e
7α-OAc	Methyl 3α-acetoxy-12-oxo-5β-cholanate	0.52	+0.36	e
	Methyl 3α,12α-diacetoxy-5β-cholanate	0.54	+0.34	e
12α-OAc	Methyl 3-oxo-5β-cholanate	0.65	+0.60	e
Cholanic acid derivative				
7α-OH	3α,12α-Dihydroxy-5β-cholanic acid	0.55	+2.23	f
	3β-Hydroxy-12-oxo-5β-cholanic acid	0.69	(+0.82)	f
12α-OH	3α,7α-Dihydroxy-5β-cholanic acid	0.55	+2.23	f
7=O	3α-Hydroxy-12-oxo-5β-cholanic acid	0.70	+0.70	f
12=O	3α,7α-Dihydroxy-5β-cholanic acid	0.55	+0.55	f
6β-OH to 6α-OH	3α,6β-Dihydroxy-5β-cholanic acid	0.46	+0.40	f
	3β,6β-Dihydroxy-5β-cholanic acid	0.42	+0.33	f

Thin-layer chromatography on silica gel (Wakogel B-5, Wako Pure Chem. Co., Tokyo) dried at 110° for 60 min.

a) Solvent system a : benzene/acetone (8:5, v/v); b : benzene/acetone (4:1, v/v); c : benzene/acetone (17:3, v/v); d : benzene/acetone (7:3, v/v); e : benzene/diethyl ether (8:1.5, v/v); f : diethyl ether/acetic acid (40:1.7, v/v)

of 1β(ax., 5β)-, 2α(eq., 5α)-, and 16α(quasi-eq.)-hydroxyl groups introduced into the respective root compounds were relatively high, that of the 11β(ax.)-hydroxyl group was moderate, and a tertiary hydroxyl group introduced into the 17α-position did not greatly enhance adsorptivity of steroids. The difference in adsorptivity between α and β configurations of the C_{20}-hydroxyl group was nearly equal to the difference between 3α- and 3β-hydroxyl groups. The fact that the 20β-alcohol is less polar than 20α-alcohol[4] is consistent with the idea of a more exposed hydroxyl group in the 20α-isomer adduced from nuclear magnetic resonance (NMR) studies.[15] Adsorption due to a carbonyl group at the 12-position was greater than for the 11-position. Both Δ^4(to 5α)- and Δ^5-double bonds made only a minor contribution to the Rm value of steroid.

15) C. H. Robinson, P. Hofer : Chem. & Ind. (London), **1966**, 377.

A difference in the Rm values for D- and L-sapogenins, *i.e.*, diastereoisomers of the methyl group at C-25, was definitely observed with the appropriate solvent system, but their mobilities were similar when unsuitable solvent systems were employed.

In the case of cholanic acid derivatives, the contribution of a ketonic group at C-3 to the Rm-function was greater than at C-7, and an acetoxyl group at the 12α-position of methyl cholanate derivatives caused more intense adsorptivity to silica gel than one at the 7α-position. Moreover, the difference in ΔRm value between 6α(eq.)- and 6β(ax.)-hydroxyl groups was very marked.

These results obtained for ΔRm values can satisfactorily be applied to the determination of the stereochemical characteristics of medium polar groups such as hydroxyl and carbonyl groups introduced into alicyclic skeletons, *e.g.*, steroids, terpenoids, and alkaloids.

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