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130. Shoji Hara*1 and Kunio Mibe*2 : Systematic Analysis of Steroids. Ⅶ.*3 Thin-layer Chromatography of Steroidal Pharmaceuticals. (1).*4

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A systematic and simultaneous analysis by thin-layer chromatography was made on thirty seven steroids used as pharmaceuticals, and the mobility and specific coloration of each compound were examined. Parameters were calculated for substituents and assumptions were made on the mechanism of the adsorption of steroid compounds.

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Upon utilization of chromatography for structural determination or characterization of a compound, it is necessary to know the quantitative relationship between the chromatographic mobility and the molecular structure.[1] Many steroids used as pharmaceuticals are modified by numerous substituents and functional groups, and their structures are so varied that they are a good subject of study for the above purpose. Although there are many chromatographic studies on natural steroids,[2] few examples[2] are known of systematic analyses of these steroidal pharmaceuticals.

About forty commercially available steroidal pharmaceuticals were selected, and systematic and simultaneous analyses by thin-layer chromatography were carried out. At the same time, the mean Rf values were determined and observations were made on the effect of substituents on the mobility.

Materials and Methods

Hydrophilic silica gel for thin-layer chromatography (Wakogel B-5, containing 5% gypsum, Wako Pure Chemical Co., Tokyo) was used as the adsorbent. This gel was stirred with two volumes of water by a mechanical stirrer to obtain a suspension, and a thin layer of 250 μ in thickness was made on a glass plate (20×20 cm.) by the use of a spreader. The thin layer was dried in air for 10 min. and activated at 110° for 60 min. Activity of the adsorbent, expressed by the mobility of pigments, gave Rf values of 0.65 for Butter Yellow and 0.11 for Indophenol (moving phase, benzene). The plate was stored in a closed vessel.

The alumina layer was made in the same manner with Alumina B-10 for thin-layer chromatography (containing 10% gypsum, Wako Pure Chemical Co., Tokyo). The thin-layer was activated by heating at 200° for 30 min.

Each steroid sample was obtained by extraction of the pharmaceutical preparation with chloroform and made into 2.0% solution in acetone. The solvent of the sample on the thin layer was evaporated under an infrared lamp.

The solvent systems used as the moving phase in the development were as follows :

a) Benzene/acetone (4:1)
b) Benzene/methanol (9:1)
c) Bush LB21/A85 [petroleum benzine (b.p. 100~120°)/benzene/glacial acetic acid/water (67:33:85:15)]

Detection reagents were (1) conc. sulfuric acid, (2) conc. sulfuric acid-acetic acid (sulfuric acid sprayed after acetic acid spray), and (3) conc. sulfuric acid with 5% vanillin added. After spraying the reagent solution, the plate was dried at 100° for 15 min.

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*3 Part Ⅵ : This Bulletin, **15**, 1032 (1967).
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1) S. Hara, M. Miyaki : This Bulletin, **15**, 1032 (1967).
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In order to obtain the correct rate of mobility, conditions outlined in the preceding paper[1] were used. Stable reproducibility of the mobility and security of an "observed" Rf value[3] measured, particularly in the "plate chromatography,"[1] very close to that of the "true" mobility,[3] were desired. Attention was paid to (1) developing temperature (16°±1°), (2) amount of sample spotted (2~3 μg.), and (3) distance developed (13 cm.), position of the starting spot (15 mm. from the lower end), and position of the immersion line (10 mm.). Development was by the ascending method. For the solvent systems (a) and (b), a new type of developing chamber[4] was used, and a horizontal type of chamber, shown in Fig. 1, for the solvent system (c). In using the latter type of chamber, biphasic solvent was placed in the chamber and the chamber was closed by a piece of adhesive tape, as shown in Fig. 1a, to be left for 3 hrs. to effect vapor saturation. Later, the development was carried out as shown in Fig. 1b, for 25 min. The samples were developed in groups (8 estrogens, 15 androgens, 3 gestagens, and 11 corticoids). Experimental values of each compound were adopted only when they fell within a range of 0.03 and the arithmetic mean of five or more replicates was calculated and converted into Rm value (=log (1/Rf−1)).[5]

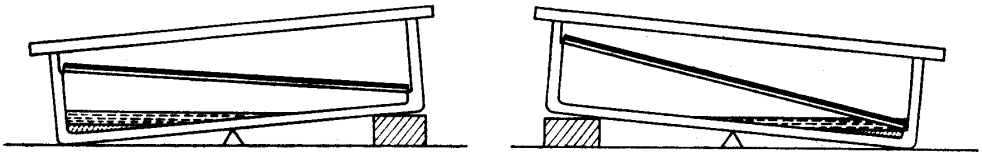

Fig. 1.

Results and Discussion

In addition to the developers (a) and (b) in adsorption system, developer (c) in partition system, generally used for paper chromatography, was employed. All of these solvent systems showed good ability for separation. Table I gives the Rf values of the samples with the three solvent systems. The samples are listed in the decreasing order of their Rf values in each group when using silica gel as the support and developed with benzene/acetone (4:1). Rf values were all obtained from the chromatogram of mixed samples. Therefore, any slight difference in the second digit of the Rf value signifies that the substances can be separated. In some cases, Rf values might be the same with one kind of a solvent system but the values would differ if the other solvent systems are used and thus the separation might be possible. All of the steroidal pharmaceutical preparations show characteristic coloration by various methods of detection (cf. Table I) and it is possible to carry out rapid and simultaneous analyses of all these thirty seven samples on the basis of the Rf value and specific coloration.

Correlation between Substituent and Mobility

Martin's theory regarding substituent and mobility applies to partition chromatography, and it seems difficult to establish this relationship in adsorption chromatography. As stated in the preceding paper,[1] however, it has been found that the parameter for a specific substituent is obtained in an approximately constant value from different kinds of compounds. Consequently, the ΔRm value for a substituent was calculated from the Rf values obtained in the present series of experiments (Table II). The ΔRm values obtained by this means were examined for a relationship between the molecular structure and adsorptivity in the case of solvent system (a) and silica gel as adsorbent.

1) The parameter of the hydroxymethylene group introduced into the position adjacent to the carbonyl group at the 3-position of the steroid (compound No. 14) is smaller than that of an ordinary hydroxyl group. This fact suggests the presence of intramolecular hydrogen bonding or adsorption as the tautomeric keto form.

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TABLE I. Rf Values and Color Reactions of Steroidal Pharmaceuticals

No.	Steroid[a]	Rf value				Color[a]					
	Adsorbent	Silica gel		Alumina	Silica gel	H_2SO_4		CH_3COOH-H_2SO_4		Vanillin-H_2SO_4	
	Solvent system	C_6H_6/Me_2CO (4:1)	C_6H_6/MeOH (9:1)	C_6H_6/Me_2CO (4:1)	Bush LB21/A85	V[a]	UV[a]	V[a]	UV[a]	V[a]	UV[a]
1	3,17β-Dipropionyloxy-E-1,3,5(10)-triene	0.75	0.78	0.68	0.63	Y R	Y G	Y R	Y	lt Y	Y
2	3-Benzoyloxy-16α,17β-diacetoxy-E-1,3,5(10)-triene	0.73	0.71	0.69	0.62	W	—	lt Y R	lt Y	lt P	dk Y
3	3-Hydroxy-17β-valeryloxy-E-1,3,5(10)-triene	0.70	0.60	0.61	0.52	dk Y	Y	Y R	Y R	R P	Y
4	3-Methoxy-17β-hydroxy-17α-Etin-E-1,3,5(10)-triene	0.66	0.52	0.60	0.57	lt R	Y R	lt R	Y R	lt B	lt Y
5	3-Hydroxy-E-1,3,5(10)-trien-17-one	0.60	0.39	0.51	0.31	Y R	Y G	Y R→R	Y	lt P	Y
6	3-Benzoyloxy-17β-hydroxy-E-1,3,5(10)-triene	0.56	0.46	0.53	0.49	dk Y R	Y	Y R	Y	dk Y	B
7	3,17β-Dihydroxy-17α-Etin-E-1,3,5(10)-triene	0.51	0.26	0.40	0.18	R	Y R	R	Y R	R P	Y
8	3,17β-Dihydroxy-E-1,3,5(10)-triene	0.39	0.17	0.41	0.19	Y	Y	—	—	lt P Y	Y
9	17β-Acetoxy-4-Cl-A-4-en-3-one	0.72	0.55	0.67	0.58	dk B	B Y R	B G	dk B	dk Y	B Y
10	17β-Acetoxy-A-4-en-3-one	0.68	0.53	0.59	0.45	G	Y G	lt G	Y G	P	R
11	17β-Enanthyloxy-A-4-en-3-one	0.64	0.68	0.67	0.52	B	B G	B	G	dk P→B	Bl
12	17β-Propionyloxy-A-4-en-3-one	0.64	0.66	0.64	0.49	B G	G	dk B	G	W	W
13	17β-Hydroxy-17α-Et-E-4-one	0.64	0.68	0.61	0.71	dk Y R	B	Y	B	lt Y	lt B
14	17β-Hydroxy-17α-Me-5α-A-3-one	0.56	0.46	0.50	0.43	lt Y R	B	lt Y R	B	dk P	Y
15	17β-Hydroxy-6α-Me-17α-(1-propynyl)-A-4-en-3-one	0.53	0.45	0.53	0.40	G	G	G	B	dk P	Y
16	17β-Hydroxy-17α-Etin-A-4-en-3-one	0.52	0.39	0.51	0.25	lt B	Y G	dkW	G	dk P	lt Y
17	17β-Hydroxy-17α-Et-E-4-en-3-one	0.52	0.40	0.48	0.31	dk Y	Y	dk Y	Y	dk Y	Y
18	17β-Hydroxy-5α,17α-diMe-A-3-one	0.52	0.49	0.53	0.32	dk Y R	B	dk Y R	B	lt dk Y	P
19	17β-Hydroxy-17α-Etin-E-4-en-3-one	0.47	0.36	0.47	0.18	lt B	G	dk Y R	G	lt W	lt Y
20	17β-Hydroxy-2-hydroxymethylene-17α-Me-5α-A-3-one	0.46	0.46	0.54	0.33	lt B	B	dk Y R	Y R	dk P→B	P
21	3β-Hydroxy-5α-A-17-one	0.45	0.37	0.48	0.26	lt B	B	dk B	Y G	lt P→B	lt P
22	17β-Hydroxy-A-4-en-3-one	0.36	0.22	0.47	0.20	B	B Y R	dk B	G	B	dk Y
23	17β-Hydroxy-17α-Me-E-4-en-3-one	0.29	0.26	0.50	0.22	lt Y	B	Y	B	lt P	B
24	P-4-ene-3,20-dione	0.61	0.59	0.60	0.39	—	G	Y R	G R	—	—
25	17α-Caproyloxy-P-4-ene-3,20-dione	0.67	0.61	0.61	0.44	dk B	R	Y R	G R	dk Y	Y
26	17α-Acetoxy-6-Cl-P-4,6-diene-3,20-dione	0.53	0.49	0.57	0.39	dk B	B Y R	dk B	Y R	dk B	dk Y
27	17α-Hydroxy-21-acetoxy-P-4-ene-3,11,20-trione	0.19	0.18	0.33	0.12	lt Y	B	W	B	lt W Y	lt B
28	11β,17α-Dihydroxy-21-acetoxy-P-4-ene-3,20-dione	0.18	0.17	0.27	0.11	lt G	G	W	G	Y B	Y B
29	11β,17α-Dihydroxy-21-acetoxy-16α-Me-6α,9α-diF-P-1,4-diene-3,20-dione	0.17	0.16	0.26	0.13	lt Y R	B	lt Y	B	dk Y	lt Y
30	11β,21-Dihydroxy-16α,17α-isopropylidenedioxy-6α,9α-diF-P-1,4-diene-3,20-dione	0.08	0.14	0.07	0.13	dk Y	B	lt Y	B	dk B	P

31	17α,21-Dihydroxy-P-4-ene-3,11,20-trione	0.08	0.13	0.06	0.11	lt Y R	B	lt Y	B	Y	lt Y B
32	17α,21-Dihydroxy-P-1,4-diene-3,11,20-trione	0.05	0.03	0.03	0.07	lt Y	B	W	G	Y	lt Y B
33	11β,17α,21-Trihydroxy-P-4-ene-3,20-dione	0.04	0.05	0.03	0.06	Y R	G	W	dk Y R	B	W
34	11β,17α,21-Trihydroxy-16β-Me-9α-F-P-1,4-diene-3,20-dione	0.03	0.04	0.02	0.03	dk Y R	—	W	dk Y R	dk Y	dk Y
35	11β,17α,21-Trihydroxy-16α-Me-9α-F-P-1,4-diene-3,20-dione	0.03	0.07	0.02	0.02	W	—	dk Y R	W	W	W
36	11β,17α,21-Trihydroxy-6β-Me-P-1,4-diene-3,20-dione	0.02	0.04	0.01	0.02	P	lt Y R	B	Y R	dk Y	dk Y
37	11β,17α,21-Trihydroxy-P-1,4-diene-3,20-dione	0.02	0.03	0.01	0.01	lt Y R	dk B	B	Y R	dk Y	Y

a) Abbreviations: Parent compounds and substituents A=androstane E=estrane P=pregnane F=fluoro Cl=chloro Me=methyl Et=ethyl Etin=ethinyl
Color B=blue G=green P=purple R=red Y=yellow W=white lt=light dk=dark Bl=black V=visible ray UV=ultraviolet ray

TABLE II. ⊿Rm Values of Converted Functional Groups of Steroids

Converted functional group	in No.	to	No. of Root Compound	Adsorbent	Silica gel				Alumina		Silica gel	
				Solvent system	C_6H_6/Me_2CO (4:1)		C_6H_6/MeOH (9:1)		C_6H_6/Me_2CO (4:1)		Bush LB21/A85	
					Rf*a*)	⊿Rm	Rf*a*)	⊿Rm	Rf*a*)	⊿Rm	Rf*a*)	⊿Rm
2=CHOH	20	14	17α Me αA 17β ol 3 one		0.56	0.175	0.46	0	0.50	−0.070	0.43	0.186
5α-CH_3	18	14	17α Me αA 17β ol 3 one		0.56	0.070	0.46	−0.053	0.50	−0.052	0.43	0.205
6β-CH_3	36	37	$P^{1,4}$ 11β,17α,21 ol 3,20 one		0.02	0	0.03	−0.130	0.01	0	0.01	−0.306
10β-CH_3	16	19	17α Etin E^4 17β ol 3 one		0.47	−0.087	0.36	−0.056	0.47	−0.069	0.18	−0.182
17α-C≡CH	7	8	$E^{1,3,5(10)}$ 3,17β ol		0.39	−0.211	0.17	−0.235	0.41	0.018	0.19	0.029
17α-C≡CH	16	22	A^4 17β ol 3 one		0.36	−0.285	0.22	−0.356	0.47	−0.069	0.20	−0.125
17α-$OCO(CH_2)_4CH_3$	25	24	P^4 3,20 one		0.61	−0.114	0.59	−0.036	0.60	−0.018	0.39	−0.089
⊿′	32	31	P^4 17α,21 ol 3,11,20 one		0.08	0.218	0.13	0.684	0.06	0.315	0.11	0.215
⊿′	37	33	P^4 11β,17α,21 ol 3,20 one		0.04	0.310	0.05	0.231	0.03	0.486	0.06	0.801
4-Cl	9	10	17β AcO A^4 3 one		0.68	−0.083	0.53	−0.035	0.59	−0.150	0.45	−0.227
11=O → 11β-OH	33	31	P^4 17α,21 ol 3,11,20 one		0.08	0.319	0.13	0.453	0.06	0.315	0.11	0.287
11=O → 11β-OH	37	32	$P^{1,4}$ 17α,21 ol 3,11,20 one		0.05	0.411	0.03	0	0.03	0.486	0.07	0.873
11=O → 11β-OH	28	27	21 AcO P^4 17α ol 3,11,20 one		0.19	0.029	0.18	0.030	0.33	0.124	0.12	0.043
17=O → 17β-OH	8	5	$E^{1,3,5(10)}$ 3 ol 17 one		0.60	0.370	0.39	0.495	0.51	0.175	0.31	0.283
3-OH → 3-$OCOC_6H_5$	6	8	$E^{1,3,5(10)}$ 3,17β ol		0.39	−0.299	0.17	−0.619	0.41	−0.210	0.19	−0.613
17β-OH → 17β-$OCOCH_3$	10	22	A^4 17β ol 3 one		0.36	−0.577	0.22	−0.602	0.47	−0.210	0.20	−0.515
17β-OH → 17β-$OCOCH_2CH_3$	12	22	A^4 17β ol 3 one		0.36	−0.500	0.22	−0.838	0.47	−0.302	0.20	−0.585
17β-OH → 17β-$OCO(CH_2)_3CH_3$	3	8	$E^{1,3,5(10)}$ 3,17β ol		0.39	−0.562	0.17	−0.865	0.41	−0.352	0.19	−0.665
17β-OH → 17β-$OCO(CH_2)_5CH_3$	11	22	A^4 17β ol 3 one		0.36	−0.500	0.22	−0.877	0.47	−0.360	0.20	−0.637
21-OH → 21-$OCOCH_3$	28	33	P^4 11β,17α,21 ol 3,20 one		0.04	−0.721	0.05	−0.590	0.03	−1.078	0.06	−0.287
3-OH → 3-OCH_3	4	7	17α Etin $E^{1,3,5(10)}$ 3,17β ol		0.51	−0.271	0.26	−0.489	0.40	−0.352	0.18	−0.781
17α-C≡CH → 17α-CH_2CH_3	17	19	17α Etin E^4 17β ol 3 one		0.47	−0.087	0.36	−0.074	0.47	−0.017	0.18	−0.312

Thin-layer chromatography on silica gel (Wakogel B-5, Wako Pure Chem. Co., Tokyo) dried at 110° for 60 min., on alumina (Alumina B-10, Wako Pure Chem. Co., Tokyo) dried at 200° for 30 min.
a) Rf value of the root compound

2) Introduction of 17α-ethinyl group decreases the adsorptivity of the 17β-hydroxyl in compounds Nos. 8 and 22. This reduction of adsorptivity is probably due to the steric hindrance of the group introduced into 17α-position in these compounds.

3) Introduction of a double bond into the root compound No. 33 (4-en-3-one system) giving No. 37 (dienone system) results in a larger Rm value. This fact indicates that the carbonyl group is the active center of adsorption and that the adsorptivity of the carbonyl group is fortified by the introduction of a double bond.

4) Reduction of the 11-oxo group in the root compound No. 31 leading to 11β-hydroxyl group results in increased adsorptivity, as would be expected.

5) Reduction of the 17-oxo group in the root compound No. 5 leading to 17β-hydroxyl group results in a fairly large parameter but acylation of the 17β-hydroxyl group in the compound No. 22 results in a negative parameter.

6) Methylation of the hydroxyl group at 3-position of the root compound No. 7 generally gives a negative parameter but the absolute value is not so large.

According to the foregoing experimental results, it is possible to carry out simultaneous qualitative analyses of steroidal pharmaceuticals by thin-layer chromatography. At the same time, ΔRm values calculated from the Rf values give some information on the adsorption mechanism of steroid compounds.

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