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The Solvent Selectivity of the Mobile Phase in Thin-Layer Chromatography in Relation to the Mobility and the Structure of Steroidal Pharmaceuticals*,[1]

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For the investigation of the solvent selectivity in liquid solid chromatography, the mobilities of various steroidal pharmaceuticals were systematically measured using silica gel and alumina thin-layer chromatography (TLC). The parameters (ΔR_M values) for the contribution of individual functional groups on the mobility were calculated by applying eleven solvent systems. The solvent selectivity was discussed on the basis of these values in relation to the structure of the steroids. For scaling the chromatographic systems examined by TLC to high performance liquid column chromatography (HPLC), their correlation in terms of mobility was extensively investigated. It was shown that the optimum system selected by TLC is applicable to HPLC with some minor modifications.

Liquid-solid chromatography (LSC) using silica gel or alumina as the packing material has developed into a basic method for the analytical separation and preparative isolation of compounds of intermediate polarity with molecular weights greater than 100—200 and less than 1000.

Among the various elution conditions with LSC, the solvent selectivity in sample separation bears a most significant role in performance. The effective choice of the mobile phase as well as the stationary phase is of particular importance.

The selected eluent provides a specific mobility R (fraction of total sample molecules in the mobile phase) for a particular sample. The characteristics of the solvent have been generally evaluated in terms of two aspects, that is the Snyder solvent strength parameter ε,[3-5] and the Hildebrand solubility parameter δ.[3,4] However, even if the sample mobility has been adjusted to the optimum value by selecting a solvent having appropriate strength, another solvent having a similar strength usually gives a different mobility for the same solute.

Up to now, the quantitative evaluation of the interaction taking part between the sample molecule and the adsorbent surface and the solvent, consequently the estimation of the solvent selectivity in chromatographic behavior has been difficult.

Efforts to solve this problem have been directed toward finding a mathematical relationship between the molecular structure of a sample and the mobility in different solvent systems. For this reason, it seems useful to measure systematically the mobilities of a group of compounds which have similar structures using various solvent systems, and subsequently calculate a parameter for the contribution of individual functional group on the mobility.

* Dedicated to the memory of Prof. Eiji Ochiai.

1) This paper constitutes Part XV of the series entitled "Systematic Analysis of Steroids" by Shoji Hara; Part XIV: *Anal. Chem.*, **40**, 1605 (1968).

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3) J.J. Kirkland, "Modern Practice of Liquid Chromatography," Wiley-Interscience, New York, 1971.

4) L.R. Snyder and J.J. Kirkland, "Introduction to Modern Liquid Chromatography," John Wiley & Sons, New York, 1974.

5) L.R. Snyder, "Principles of Adsorption Chromatography," Marcel Dekker, New York, 1964.

Steroidal pharmaceuticals seem to be suitable samples for such a chromatographic investigation because they contain various functional groups and many positional isomers and stereoisomeric derivatives. However, only a few systematic studies concering LSC of such compounds have been reported.[6-8]

While the efficiency of high performance liquid column chromatography (HPLC) is very high, the range of capacity factor k',[9] in which solutes can be analyzed simultaneously in HPLC is rather narrow and indicated as $0>k'>10$.[3] On the other hand with thin-layer chromatography (TLC), this range is wider and increases to $0>k'>100$.[10] Therefore, it should be convenient to use TLC for such investigations.

On the basis of these considerations, systematic studies of steroidal pharmaceuticals using TLC in terms of solvent selectivity have been carried out.

Herein, it should be noted that TLC has a flat bed[11] and a dry column. As a result, several characteristic phenomena have been observed, *i.e.*, the volume of mobile phase, V_m, varies with respect to the height of the thin-layer plate, preadsorption of solvent vapor and solvent demixing problems occur, *etc.*[12,13] Therefore, these differences and their relationship between HPLC having a wet column and TLC were extensively investigated in order to apply effectively the mobility correlations obtained from TLC data to HPLC systems.

Materials and Methods

Silica gel (Wakogel B-5, containing 5% gypsum, Wako Pure Chemicals Co., Osaka), alumina (Alumina B-10, containing 10% gypsum, Wako Pure Chemicals Co.) were used as adsorbents for preparation of the thin-layer plates.

Eleven chromatographic systems were developed for the TLC of these compounds.

I silica gel–*n*-hexane/ethyl acetate (2: 8 v/v)
II silica gel–benzene/ethyl acetate (3: 7 v/v)
III silica gel–diethyl ether
IV silica gel–benzene/acetone (4: 1 v/v)
V silica gel–chloroform (containing 1% ethanol as a stabilizer)/acetone (3: 1 v/v)
VI silica gel–benzene/methanol (9: 1 v/v)
VII silica gel–chloroform/methanol (97: 3 v/v)
VIII silica gel–chloroform/acetic acid (3: 1 v/v)
IX silica gel–Bush LB21/A85[petroleum benzine (b p 100—120°)/benzene/acetic acid/water (67: 33: 85: 15 v/v)]
X alumina–benzene/acetone (4: 1 v/v)
XI alumina–benzene/methanol (9: 1 v/v)

All thin-layer chromatographic procedures were carried out under conditions described previously.[6,8]

Silica gel used as packing materials of HPLC was obtained by fractionation of the products for TLC (Wakogel B-0, Wako Pure Chemicals Co.) applying wet sieving. A fraction having 15—25 μ of particle size was collected. It was activated at 100°, 1 hour. Glass columns having 5 mm inner diameter, 300 mm in length were filled with silica gel by using the dry packing procedure.

Steroid samples from 0.2 to 0.5 mg were dissolved in 5 μl of methylene chloride, and injected by using a 10 μl of syringe (Kusano Scientific Co., Tokyo) on the column applying a stop flow technique.

A reciprocating piston pump (Kyowa Seimitsu KSD-45) was used. Solvents were delivered at a flow rate of 1 ml/min., at 10 to 30 kg/cm² inlet pressure.

A Waters refractoindex detector RI-401 (Waters Associates) and an electric recorder (Ohkura Electric Co., Tokyo) were used to record the elution data.

6) S. Hara and K. Mibe, *Chem. Pharm. Bull.* (Tokyo). **15**, 1036 (1967).
7) S. Hara and K. Mibe, *The Annual Report of the Tokyo College of Pharmacy*, **18**, 308 (1968).
8) S. Hara and K. Mibe, *Anal. Chem.*, **40**, 1605 (1968).
9) Capacity factor $k'=(V_R-V_0)/V_0=1/R-1$ V_R, V_0: indicated in Table III.
10) Waters report AN 103, Waters Associates, Massachusetts (1971).
11) I.M. Hais, *J. Chromatog.*, **94**, 353 (1974).
12) S. Hara and K. Mibe, *J. Chromatog.*, **66**, 75 (1972).
13) S. Hara and K. Mibe, *Chimia*, **24**, 39 (1970).

Results and Discussion

Thirty six synthetic steroids were investigated including pairs of compounds which contain the same partial structure differing only in the presence of a characteristic functional group, *e.g.* 6β-hydroxytestosterone (**26**) and testosterone (**21**). Silica gel and alumina were used as adsorbents, and most solvent systems which were prepared were able to analyze all of the samples simultaneously on a single thin-layer plate. For cortico-steroids which have larger polarities, an additional mobile phase was applied.

In the LSC of steroids, it is difficult to find a single solvent which has the right solvent strength other than in the case of diethyl ether. Thus mixed solvent systems were prepared. A polar solvent was added to another solvent which has low solvent strength such as *n*-hexane, benzene or chloroform. It has been pointed out that the selectivity of a mobile phase affecting mobilities in LSC are mainly made by the polar constituents.[3] In this study, ethyl acetate, acetone, methanol were chosen as the polar solvent. Eight solvent systems for silica gel and two for alumina were thus examined. Moreover, a water containing mobile phase for silica gel, recommended by Bush for paper chromatography,[14] was also selected.

In order to obtain reproducible values for mobility, chromatographic procedures were carried out under complete preadsorption of solvent vapor by the adsorbents.[12] Such preconditioning is very useful, because this seems to be most similar to HPLC wet columns. A new sandwich type developing chamber with a characteristic cover plate, inside of which filter paper permeated with solvent was attached,[12] was used to insure complete preadosorption of the solvent vapor.

Moreover, to obtain reproducible results, four dyestuffs which have similar mobilities as the steroid samples were chosen as internal standards. All R_F values are shown in Table I.

To evaluate the contribution of a functional group toward the chromatographic behavior, the useful, known procedure was followed wherein R_M values[15] were derived from R_F values, and the ΔR_M value was calculated for a specific functional group from the R_M values of a pair of steroid samples.[6,8] Such values seem to be roughly related with the adsorption energy, and in accord with the additivity rule. The ΔR_M values calculated for the functional groups are shown in Table II.

According to the parameters of functional groups which were obtained above, the selectivity of developing solvents for particular functional group will be discussed as follows.

Secondary or tertiary hydroxyl groups introduced into the 6β, 14α or 16α position of the steroids gave generally large positive ΔR_M values. Such values for the 14α, 16α-hydroxyl groups were almost always larger than that for 6β-hydroxyl group, since the latter group is in a 1,3-diaxial relationship with 10β-methyl group.

In the chromatographic systems, silica gel–ethyl acetate/*n*-hexane or benzene (I or II), the parameters were smaller than in other systems. The ΔR_M values for the systems of silica gel-methanol(VI, VII) were relatively small. These facts seem to be attributable to the solvation of steroid molecules. In former case, ethyl acetate may interact as proton acceptor, and in latter case, methanol takes part in as proton donor. On the other hand, in the case of alumina-benzene/acetone (X), large parameters were generally obtained.

Acylation of 17β- and 21-hydroxyl group always gave large negative ΔR_M values. The absolute values of ΔR_M for the homologous series of the acyl group from $-COCH_3$ to $-CO-(CH_2)_5CH_3$ tend to increase with increasing number of carbons.

Although the trends for parameters of the acylation for any chromatographic system were similar, the system of silica gel–ethyl acetate/*n*-hexane or benzene (I or II) afforded smaller negative ΔR_M values. This suggests that the solvation of ethyl acetate with the

14) I.E. Bush, "The Chromatography of Steroids" Pergamon Press, Oxford, 1961; D.M. Cathro, J. Cameron, and K. Birchall, *J. Chromatog.*, **17**, 362 (1965).

15) R_M value: $R_M = \log(1/R_F - 1)$.

TABLE I. R_F Values of Steroidal Pharmaceuticals

No.	Steroid[a]	Adsorbent / Solvent system	I *n*-hexane/EtOAc (2:8)		II C_6H_6/EtOAc (3:7)		III ether		IV C_6H_6/acetone (4:1)	
			R_F	S.D.[b]	R_F	S.D.	R_F	S.D.	R_F	S.D.
1	3-Hydroxy-17β-valeroyloxy-E-1,3,5(10)-triene		0.75	0.02	0.77	0.01	0.77	0.02	0.70	0.02
2	3-Methoxy-17β-hydroxy-17α-Etin-E-1,3,5(10)-triene		71	0.01	72	0.02	76	0.03	66	0.02
3	3-Hydroxy-E-1,3,5(10)-trien-17-one		66	0.01	67	0.02	67	0.02	60	0.02
4	3-Benzoyloxy-17β-hydroxy-E-1,3,5(10)-triene		62	0.02	61	0.02	59	0.02	56	0.02
5	3,17β-Dihydroxy-17α-Etin-E-1,3,5(10)-triene		68	0.02	68	0.02	72	0.03	51	0.02
6	3,17β-Dihydroxy-E-1,3,5(10)-triene		59	0.03	59	0.02	55	0.03	39	0.03
7	3,16α,17β-Trihydroxy-E-1,3,5(10)-triene		21	0.03	16	0.02	07	0.03	05	0.03
8	17β-Acetoxy-4-Cl-A-4-en-3-one		71	0.02	68	0.02	72	0.03	72	0.01
9	17β-Acetoxy-A-4-en-3-one		64	0.02	62	0.04	59	0.02	68	0.01
10	17β-Enanthoxy-A-4-en-3-one		74	0.03	67	0.02	70	0.04	64	0.02
11	17β-Propionyloxy-A-4-en-3-one		67	0.02	61	0.03	69	0.03	64	0.02
12	17β-Hydroxy-17α-Et-E-4-ene		74	0.02	66	0.03	73	0.03	64	0.03
13	17β-[β-(Phenylpropyonyloxy)]-E-4-en-3-one		69	0.02	68	0.02	63	0.03	58	0.01
14	17β-[β-(2-Furylpropyonyloxy)]-E-4-en-3-one		69	0.02	68	0.02	63	0.04	58	0.01
15	17β-Hydroxy-17α-Me-5α-A-3-one		60	0.03	57	0.03	56	0.04	56	0.02
16	17β-Hydroxy-5α-A-3-one		59	0.02	53	0.02	56	0.02	53	0.03
17	17β-Hydroxy-17α-Etin-A-4-en-3-one		53	0.02	55	0.03	50	0.03	52	0.02
18	17β-Hydroxy-17α-Et-E-4-en-3-one		53	0.03	46	0.02	35	0.02	52	0.02
19	17β-Hydroxy-17α-Etin-E-4-en-3-one		51	0.02	52	0.01	50	0.03	47	0.02
20	17β-Hydroxy-2-hydroxymethylene-17α-Me-5α-A-3-one		43	0.02	54	0.02	13	0.02	46	0.01
21	17β-Hydroxy-A-4-en-3-one		45	0.02	41	0.02	32	0.03	36	0.03
22	17β-Hydroxy-17α-Me-E-4-en-3-one		51	0.03	39	0.02	38	0.03	35	0.04
23	17β-Hydroxy-E-4-en-3-one		41	0.02	36	0.02	39	0.04	34	0.03
24	17β-Hydroxy-17α-Me-A-1,4-dien-3-one		40	0.04	50	0.02	31	0.02	27	0.02
25	14α-Hydroxy-A-4-ene-3,17-dione		34	0.03	31	0.02	12	0.02	20	0.02
26	6β,17β-Dihydroxy-A-4-en-3-one		29	0.02	25	0.01	17	0.02	13	0.03
27	14α,17β-Dihydroxy-A-4-en-3-one		18	0.02	16	0.02	07	0.02	09	0.03
28	P-4-ene-3,20-dione		62	0.02	59	0.02	58	0.02	61	0.03
29	17α-Caproyloxy-P-4-ene-3,20-dione		67	0.02	63	0.02	68	0.02	67	0.03
30	17α-Hydroxy-21-acetoxy-P-4-ene-3,11,20-trione		49	0.02	41	0.02	23	0.01	19	0.04
31	11β,17α-Dihydroxy-21-acetoxy-P-4-ene-3,20-dione		48	0.03	40	0.02	29	0.02	18	0.03
32	17α,21-Dihydroxy-P-4-ene-3,11,20-trione		33	0.02	22	0.02	08	0.02	08	0.03
33	17α,21-Dihydroxy-P-1,4-diene-3,11,20-trione		35	0.02	19	0.02	14	0.02	05	0.02
34	11β,17α,21-Trihydroxy-P-4-ene-3,20-dione		27	0.02	18	0.01	08	0.02	04	0.03
35	11β,17α,21-Trihydroxy-6β-Me-P-1,4-diene-3,20-dione		26	0.02	17	0.02	07	0.02	02	0.02
36	11β,17α,21-Trihydroxy-P-1,4-diene-3,20-dione		24	0.03	15	0.02	06	0.02	02	0.02
	Dimethylaminoazobenzene		71	0.02	66	0.02	80	0.01	71	0.02
	Isatin		55	0.02	47	0.02	50	0.02	40	0.02
	Neutral red		05	0.01	05	0.02	00		06	0.02
	Indophenol									

a) parent compounds and substituents: A=androstane, E=estrane, P=pregnane, Cl=chloro, Me=methyl, Et=ethyl, Etin=ethinyl
b) S.D.=standard deviation of 10 runs

Silica gel										Alumina			
V $CHCl_3$/acetone (3:1)		VI C_6H_6/MeOH (9:1)		VII $CHCl_3$/MeOH (97:3)		VIII $CHCl_3$/HOAc (3:1)		IX Bush LB21/A85		X C_6H_6/acetone (4:1)		XI C_6H_6/MeOH (9:1)	
R_F	S.D.	R_F	S.D.	R_F	S.D.	R_F	S.D.	R_F	S.D.	R_F	S.D.	R_F	S.D.
0.64	0.03	0.60	0.03	0.53	0.02			0.52	0.01	0.61	0.04	0.60	0.04
61	0.03	52	0.02	57	0.02			57	0.01	60	0.04	64	0.03
58	0.02	39	0.02	32	0.05			31	0.03	51	0.02	50	0.05
56	0.03	46	0.02	44	0.06			49	0.03	53	0.03	63	0.03
51	0.02	26	0.02	22	0.02			18	0.03	40	0.04	28	0.03
42	0.02	17	0.03	18	0.02			19	0.03	41	0.07	43	0.05
08	0.02	08	0.02	03	0.03			18	0.02	06	0.04	09	0.04
69	0.02	55	0.03	66	0.02			58	0.02	67	0.02	68	0.06
66	0.03	53	0.02	50	0.04			45	0.02	59	0.03	65	0.04
69	0.02	68	0.04	67	0.02			52	0.02	67	0.03	68	0.04
66	0.02	66	0.03	57	0.02			49	0.01	64	0.03	67	0.04
60	0.02	68	0.02	66	0.01			71	0.02	61	0.02	76	0.06
62	0.02	57	0.02	56	0.03			52	0.01	60	0.03	74	0.04
62	0.02	57	0.02	56	0.03			52	0.01	60	0.03	74	0.04
55	0.03	46	0.03	40	0.04			43	0.02	50	0.04	59	0.04
50	0.01	46	0.03	35	0.02			41	0.02	46	0.05	56	0.07
53	0.02	39	0.02	32	0.03			25	0.02	51	0.04	60	0.04
52	0.02	27	0.02	29	0.02			31	0.02	48	0.03	52	0.04
50	0.02	36	0.02	31	0.01			18	0.02	47	0.02	44	0.03
34	0.03	46	0.03	29	0.02			33	0.01	54	0.04	02	0.03
41	0.02	22	0.03	25	0.04			20	0.02	47	0.06	43	0.03
47	0.02	26	0.04	29	0.02			22	0.02	50	0.03	51	0.06
36	0.01	21	0.02	28	0.02			14	0.02	39	0.04	49	0.05
36	0.01	15	0.04	26	0.03			15	0.02	23	0.03	39	0.03
32	0.01	09	0.03	14	0.02			07	0.02	12	0.03	34	0.03
18	0.02	06	0.02	09	0.02			05	0.02	07	0.03	16	0.03
09	0.02	05	0.02	06	0.02			03	0.02	05	0.04	14	0.04
60	0.02	59	0.02	66	0.02			39	0.02	60	0.05	70	0.04
63	0.03	61	0.03	67	0.02			44	0.02	61	0.05	72	0.05
44	0.03	18	0.02	22	0.03	0.43	0.01	12	0.03	33	0.04	58	0.04
33	0.02	17	0.03	14	0.03	32	0.01	11	0.02	27	0.03	48	0.03
17	0.02	13	0.03	07	0.02	19	0.01	11	0.02	06	0.04	19	0.04
15	0.02	03	0.04	05	0.02	16	0.02	07	0.02	03	0.03	17	0.02
10	0.02	05	0.03	03	0.01	11	0.01	06	0.03	03	0.02	10	0.03
09	0.03	04	0.03	02	0.02	11	0.01	02	0.03	01	0.03	09	0.03
09	0.03	03	0.04	02	0.02	11	0.01	01	0.02	01	0.04	09	0.03
70	0.02	74	0.02	82	0.02	69	0.01	37	0.03	75	0.03	79	0.04
45	0.03	25	0.02	13	0.03	68	0.01	08	0.02	43	0.03		
07	0.03	25	0.02	07	0.02	05	0.01			25	0.03		
						71	0.01						

TABLE II. ΔR_M Values of Converted Functional Groups of Steroids

Converted functional group	in No.	to No. of basic steroid	Adsorbent: Silica gel — Solvent system: I *n*-hexane/EtOAc (2:8)	II C_6H_6/EtOAc (3:7)	III ether	IV C_6H_6/acetone (4:1)	V $CHCl_3$/acetone (3:1)	VI C_6H_6/MeOH (9:1)	VII $CHCl_3$/MeOH (97:3)	VIII $CHCl_3$/HOAc (3:1)	IX Bush LB21/A85	Alumina: X C_6H_6/acetone (4:1)	XI C_6H_6/MeOH (9:1)
6β-OH	26	21	0.30	0.32	0.36	0.58	0.50	0.65	0.53		0.68	1.07	0.60
14α-OH	27	21	0.57	0.56	0.80	0.76	0.85	0.73	0.72		0.91	1.23	0.67
16α-OH	7	6	0.73	0.88	1.21	1.09	0.92	0.37	0.85		0.03	1.04	0.88
17β-OH→17β-$OCOCH_3$	9	21	−0.34	−0.37	−0.49	−0.58	−0.45	−0.60	−0.48		−0.52	−0.21	−0.39
17β-OH→17β-$OCOCH_2CH_3$	11	21	−0.40	−0.35	−0.68	−0.50	−0.45	−0.84	−0.60		−0.59	−0.30	−0.43
17β-OH→17β-$OCO(CH_2)_3CH_3$	1	6	−0.32	−0.37	−0.44	−0.56	−0.39	−0.87	−0.71		−0.67	−0.35	−0.30
17β-OH→17β-$OCO(CH_2)_5CH_3$	10	21	−0.54	−0.47	−0.70	−0.50	−0.51	−0.88	−0.79		−0.64	−0.36	−0.45
17β-OH→17β-$OCOCH_2CH_2C_6H_5$	13	23	−0.51	−0.58	−0.43	−0.43	−0.46	−0.68	−0.52		−0.82	−0.37	−0.47
17β-OH→17β-$OCOCH_2CH_2C_4H_3O$	14	23	−0.51	−0.58	−0.43	−0.43	−0.46	−0.68	−0.52		−0.82	−0.37	−0.47
21-OH→21-$OCOCH_3$	31	34	−0.40	−0.48	−0.67	−0.72	−0.65	−0.59	−0.72	−0.58	−0.29	−1.08	−0.92
3-OH→3-OCH_3	2	5	−0.06	−0.08	−0.09	−0.27	−0.18	−0.49	−0.67		−0.78	−0.35	−0.66
3-OH→3-$OCOC_6H_5$	4	6	−0.06	−0.04	−0.07	−0.30	−0.25	−0.62	−0.55		−0.61	−0.21	−0.35
17α-CH_3	15	16	−0.02	−0.07	0	−0.05	−0.09	0	−0.09		−0.04	−0.07	−0.05
17α-CH_3	22	23	−0.18	−0.06	0.02	−0.02	−0.20	−0.12	−0.02		−0.24	−0.19	−0.03
17α-C_2H_5	18	23	−0.21	−0.18	0.08	−0.32	−0.29	−0.14	−0.02		−0.44	−0.16	−0.05
17α-C≡CH	19	23	−0.18	−0.29	−0.19	−0.24	−0.25	−0.33	−0.06		−0.13	−0.14	−0.09
17α-C≡CH	5	6	−0.17	−0.17	−0.32	−0.21	−0.16	−0.24	−0.11		−0.03	−0.02	−0.29
17α-C≡CH	17	21	−0.14	−0.24	−0.33	−0.29	−0.21	−0.36	−0.15		0.13	0.07	0.30
17α-C≡CH→17α-C_2H_5	18	19	0	0	0.27	−0.09	−0.04	0.18	0.04		−0.31	−0.02	−0.14
C_2=CHOH	20	15	0.30	0.05	0.93	0.18	0.38	0	0.21		0.19	−0.07	1.85
C_{11}=O→11β-OH	34	32	0.12	0.11	0	0.32	0.27	0.45	0.39	0.28	0.29	0.32	0.32
C_{11}=O→11β-OH	36	33	0.19	0.06	0.34	0.41	0.25	0	0.41	0.19	0.57	0.49	0.32
C_{11}=O→11β-OH	31	30	0.02	0.02	−0.14	0.03	0.20	0.03	0.24	0.21	0.04	0.12	0.18
C_{17}=O→17β-OH	6	3	0.13	0.15	0.22	0.37	0.28	0.50	0.33		0.28	0.18	0.12
C_{17}=O→17β-OH	27	25	0.37	0.37	0.26	0.40	0.68	0.27	0.41		0.39	0.41	0.50
C_3=O	18	12	0.40	0.36	0.70	0.22	0.14	0.76	0.68		0.74	0.23	0.47
5α-H→Δ^4	21	16	0.26	0.23	0.43	0.30	0.16	0.53	0.58		0.44	−0.02	0.34
5α-H→$\Delta^{1,4}$	24	15	0.35	0.12	0.45	0.54	0.34	0.68	0.28		0.63	0.53	0.35
4-Cl	8	9	−0.14	−0.11	−0.25	−0.08	−0.06	−0.04	−0.29		−0.23	−0.15	−0.06
Δ^1	33	32	−0.04	0.08	−0.27	0.22	0.06	0.68	0.16	0.09	0.22	0.32	0.06
Δ^1	36	34	0.02	0.03	0.06	0.31	0.05	0.10	0.18	0	0.50	0.49	0.05
17α-$OCO(CH)_{24}CH_3$	29	28	−0.10	−0.07	−0.19	−0.11	−0.06	−0.04	−0.02		−0.09	−0.02	−0.04
6β-CH_3	35	36	−0.05	0.06	0.07	0	0	−0.13	0	0	−0.31	0	0
10β-CH_3	17	19	−0.04	−0.05	0	−0.09	−0.05	−0.06	−0.03		−0.18	−0.07	−0.28
10β-CH_3	21	23	−0.07	−0.09	0.13	−0.09	−0.09	−0.03	0.07		−0.19	−0.14	0.11

hydroxyl group decreases their adsorptivity of the basic steroid structure. Using the silica gel–methanol containing solvent systems (VI, VII) and the Bush system (IX), uusally larger negative values were observed than in the case of the acetone containing solvent systems (IV, V).

Alkylation or acylation of phenolic hydroxyl group at position 3 gave wide range of parameters according to the differences of the solvent systems. The absolute values of ΔR_M obtained by using solvent systems including diethyl ether (III) or ethyl acetate containing solvents (I, II) were particularly small. The results may indicate that the formation of strong hydrogen bonding between one of these solvents as proton acceptor and the phenolic hydroxyl group especially weakens the adsorption of the steroid. On the contrary, however, methanol containing solvent systems (VI, VII) or the Buch system including acetic acid (IX) gave extremely large absolute values of ΔR_M.

Examination of the influence of methyl, ethyl and ethinyl groups substituted at the 17α-position of steroids on the adsorptivity of 17β-hydroxyl group, showed that the introduction of these groups afforded negative ΔR_M values as was expected. The absolute ΔR_M values of 17α-methyl group were less than 0.1 with exception of a few cases. However, the 17α-ethyl and ethinyl groups gave relatively large parameters, which indicated that these bulky groups interfere with the adsorption of the 17β-hydroxyl group. Although the difference of ΔR_M values between ethyl and ethinyl groups was quite small, a distinct difference was clearly observed in the case of silica gel–diethyl ether (III) or benzene/methanol (VI) and the Bush solvent (IX).

Parameters of the hydroxymethylene group at 2 position of 3-oxo-steroids varied widely in these chromatographic systems. Silica gel–diethyl ether (III), alumina–benzene/methanol (XI) gave extremely large values. This fact suggests that the hydroxyl tautomeric form takes part in adsorption of the steroid. However, other systems gave small values ranging from 0 to 0.38, in which it was assumed that there was intermolecular hydrogen bonding between enol form of the hydroxymethylene group and 3-carbonyl group, or the adsorption of formyl group at 2 position as a carbonyl form.

Reduction of the carbonyl group at the 11 or 17 position of the steroid into the corresponding β-hydroxyl group gave wide range of parameters from -0.14 to 0.68. Generally, the parameters for the 17 position were larger than those for the 11 position in the steroid structures.

Parameters of the carbonyl group at position 3 varied extensively according to the solvent system. Silica gel–methanol containing solvents (VI, VII) or the Bush system (IX) and diethyl ether (III) afforded large ΔR_M values.

Introduction of double bond at position 4 or two double bonds at the 1 and 4 positions of 3-oxo-steroids, resulted in comparatively large parameters. This fact seems to be due to the electronic effect of conjugation. Using silica gel-methanol containing solvent (VI, VII) or the Bush solvent (IX), especially large ΔR_M values were observed. However, in the case of the ethyl acetate containing solvent (I, II), rather small ΔR_M values were obtained.

Introduction of chlorine at 4 position of the 3-oxo-Δ^4-steroids gave negative parameters, wherein the decrease of adsorptivity of the carbonyl group was assumed due to the inductive effect of chlorine. Larger negative parameters were obtained by using silica gel-chloroform/methanol (VII), diethyl ether (III) or the Bush system (IX).

Dehydrogenation of the 3-oxo-Δ^4-steroids to introduce double bond at 1 position generally gave positive parameters, which were explained by the effects of conjugation.

Acyloxyl group introduced into the 17α-position which is adjacent to the carbonyl group at the 20 position gave negative parameters. However, absolute values of ΔR_M were always small with the exception of the use of diethyl ether (III).

Methyl groups introduced into 6β or 10β of the 3-oxo-Δ^4-steroids generally gave negative ΔR_M values. They weaken the adsorptivity of the oxo-steroids, which was probably due

to the steric hindrance of the methyl group. Such ΔR_M values varied with respect to the solvent systems. The most definitive values were obtained in the case of the Bush system (IX).

All the results described above can be summerized as follows.

Using silica gel–ethyl acetate/*n*-hexane or benzene systems (I or II), comparatively small ΔR_M values were obtained. This indicates that the capacity factors for these systems were distributed in a relatively narrow range of values. Therefore, ethyl acetate containing solvent systems were found to be useful for simultaneous separation of a variety of compounds having intermediate polarities like steroids.

Silica gel–diethyl ether system (III) gave characteristic parameters which differ from those obtained by using other solvent systems. It seems to be useful for its remarkable solvent selectivity.

Moreover, silica gel–methanol/benzene or chloroform (VI or VII) and silica gel–Bush system (IX) gave also characteristic parameters. These features seem to be due to the hydrogen bonding ability of methanol or acetic acid, and their solvation phenomena. It was observed that while these solvents sometimes weakened the adsorption of the alcoholic hydroxyl group, they strengthened that of the phenolic hydroxyl group or carbonyl group.

Subsequently, chromatographic behaviors on HPLC were examined for comparison with those in TLC. For the clarification of such relationships, the TLC adsorbent seemed suitable as a packing material for HPLC in this study. However, the particle size distribution of TLC adsorbents which are commercially available is rather too wide for direct application to HPLC column packing. Therefore, it seemed necessary to size the particles of the TLC adsorbent product. The fraction having the particle size from 15 to 25 μ is used in this study.

For practical HPLC separations, a rather narrow range of capacity factors are usually chosen. In this investigation using the same solvent systems which were selected for TLC, steroids having capacity factors from 1 to 2, namely R_F values from 0.3 to 0.5 seem to be suitable solutes for chromatography. Three steroids (**18**, **21** and **23**) and isatin were thus selected, and silica gel with the seven solvents described above were applied as chromatographic systems.

Retention volumes V_R, mobilities R which were obtained by performing HPLC, are shown in Table III.

In the case of the systems such as ethyl acetate/*n*-hexane or benzene (I or II), and diethyl ether (III), acetone/benzene or chloroform (IV or V), R values for HPLC were about 1.5 times of the TLC R_F values. Following these results, it was concluded that TLC-HPLC relationship is rather simple and finding proper experimental conditions for transferring the data is not difficult for these solvents.

However, it was found that the solvent systems of methanol/benzene or chloroform (VI or VII) gave particularly large R values for HPLC. In this respect, modified mobile phases such as benzene/methanol (95: 5), (98: 2), (99: 1) and chloroform (containing 1% ethanol)/methanol (99: 1) were prepared. The results obtained by these solvent systems are also presented in Table III.

In the case of benzene/methanol, R_F values for TLC in the ratio of 90: 10 were similar to the R values for HPLC in the ratio of 98: 2 or 99: 1. For the chloroform/methanol system, R_F values for TLC in the ratio of 97: 3 was smaller than R values for HPLC using chloroform/methanol (99: 1) and chloroform.

Such remarkable differences found in the results obtained by using methanol containing systems seem to be due to preadsorption effects of the solvent vapor and the demixing phenomenon of the solvent systems in the dry TLC column bed.

Considering the relationships between TLC and HPLC described above, the chromatographic separation process can be simplified as follows.

On the basis of known structural information, the contributions of given functional groups on the mobility and solvent selectivity can be estimated. A mobile phase having an optimum solvent selectivity and strength would then be selected. By using TLC as a pretest for examin-

TABLE III. Retention Volumes and Mobilities for HPLC Corresponding R_F Values for TLC of the Steroids

Solvent system	I *n*-hexane/EtOAc (2:8 v/v)			II C_6H_6/EtOAc (3:7 v/v)			III ether			IV C_6H_6/acetone (4:1 v/v)			V $CHCl_3$/acetone (3:1 v/v)			VI C_6H_6/MeOH (9:1 v/v)	
No. of steroid	$V_R$ml	R[a]	R_F	$V_R$ml	R	R_F	$V_R$ml	R	R_F	$V_R$ml	R	R_F	$V_R$ml	R	R_F	R	R_F
23	10.6	0.52	0.41	9.8	0.57	0.36	12.0	0.46	0.39	11.0	0.52	0.34	8.6	0.65	0.36	—[b]	0.21
21	9.6	0.57	0.45	9.3	0.60	0.41	11.2	0.49	0.32	10.8	0.53	0.36	8.2	0.68	0.41	—[b]	0.22
18	8.2	0.67	0.53	8.1	0.69	0.46	10.4	0.53	0.35	8.5	0.67	0.52	7.4	0.76	0.52	—[b]	0.27
Isatin	7.2	0.76	0.53	7.1	0.79	0.51	8.5	0.65	0.45	9.2	0.62	0.40	7.6	0.74	0.42	—[b]	0.25
V_0[c]	5.5			5.6			5.5			5.7			5.6				

Solvent system	C_6H_6/MeOH (95:5 v/v)		C_6H_6/MeOH (98:2 v/v)		C_6H_6/MeOH (99:1 v/v)		VII $CHCl_3$/MeOH (97:3 v/v)			$CHCl_3$/MeOH (99:1 v/v)		$CHCl_3$	
No. of steroid	$V_R$ml	R	$V_R$ml	R	$V_R$ml	R	$V_R$ml	R	R_F	$V_R$ml	R	$V_R$ml	R
23	9.3	0.61	17.1	0.34	—[d]	—[d]	8.2	0.73	0.28	9.7	0.59	18.4	0.31
21	9.0	0.63	16.1	0.36	—[d]	—[d]	8.0	0.75	0.28	9.4	0.62	16.8	0.34
18	—[b]	—[b]	12.3	0.47	26.2	0.21	—[b]	—[b]	0.29	7.9	0.73	13.0	0.44
Isatin	9.5	0.59	15.7	0.37	28.0	0.19	9.2	0.65	0.20	12.3	0.47	24.8	0.23
V_0[c]	5.7		5.8		5.5		6.0			5.8		5.7	

a) $R=V_0/V_R$
b) The peak was not detected because of apparent overlap with the peak of which solvent injected.
c) V_0: Volume of mobile phase within column, out side of particles.
d) The peak was not detected baecuse of apparent broadening.

ing the predicted chromatographic behavior, a solvent system and the solvent strength will be determined. After modifying the ratio of the solvent systems according to the relationship observed above, the corrected chromatographic systems are applicable to HPLC.

Using the TLC preexamination and HPLC performance, it was possible to simply and efficiently purify about 30 mg of a steroid in approximately 30 minutes from a sample containing a few impurities.

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