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239. Toshio Kawasaki and Kazumoto Miyahara: Thin Layer Chromatography of Steroid Saponins and their Derivatives.*1

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Thin layer chromatography is a versatile method in separating various kinds of complex mixtures and has many advantages over paper chromatography. Its applications to cardiac glycosides,[1] steroid alkaloids,[2] methylated glycosides,[3] carbohydrate acetates,[4] steroid sapogenins[5] and triterpenoids[6] have been reported, but no study in the field of steroid and triterpenoid saponins, with the exceptions of several unsystematic applications,[7,8] is found in the literatures.

In our study on the steroid saponins, paper chromatography[9] has served as a convenient method to separate mixtures, to determine the purity and to identify the substance. However, the method failed to separate some closely related saponins, for example, tetraglycosides in Digitalis seeds,[10] and leaves,[10] and the slightly polar saponin derivatives such as acetates and methylates were not resolved on the paper chromatograms. An application of thin layer chromatography to steroid saponins and their derivatives has therefore been undertaken.

Chromatoplates employed were glass plates coated with silica gel containing gypsum at a thickness of 250 microns. Standard compounds examined were thirteen kinds of saponins previously obtained from the plants of *Dioscorea*, *Anemarrhena*, and *Digitalis* genera (Table I), one steroid alkaloid (tomatine) of which structure[11] is closely related to desgalactotigonin,[10] nine kinds of their peracetates and four of permethylates (Table II). All saponins were homogeneous in paper chromatographic examinations[9] and their compositions (kind and number of sugars and aglycone) or structures had been established. Peracetates and permethylates had been prepared, respectively, by acetylation

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11) R. Kuhn, I. Löw, H. Trischmann: Chem. Ber., **86**, 1072 (1953); **90**, 203 (1957).

with acetic anhydride-pyridine at room temperature and by repeated methylation according to the Kuhn's method[12] and showed no hydroxyl absorption in the infrared spectra. Several kinds of solvents were tried with the reference to those used for cardiac glycosides[1] and steroid sapogenins[5] and butanol saturated with water, the upper phase of butanol-acetic acid-water (4:1:5) mixture, the lower phase of chloroform-methanol-water (65:35:10) for highly polar saponins, chloroform-methanol (80:20) mixture for less polar ones, chloroform-ethanol (100:2) for acetates and benzene-acetone (80:20) for methylates were found to give satisfactory results. Developments were completed in a relatively short time and the chromatograms were visualized by spraying with a 10% solution of sulfuric acid followed by heating to give a sharp spot. Less than 1 γ of sample could be detected and the color of a spot varied depending on the structure of the aglycone suggesting the kind of the component sapogenin.[5b] The Rf value of each authentic saponin is given in Table I.

A mixture of saponins was completely resolved as illustrated in Fig. 1a. The order of mobilities of most saponins on the thin layers were, as in the paper chromatography, in inverse proportion to the total number of hydroxyl group they hold in the sugar and aglycone moieties and the chromatogram of a sample run in pararell with known saponins seemed to suggest the number of the component sugars of the compound. In particular, an attention was drawn to the findings that a mixture of gitonin and desglucodigitonin or of F-gitonin and desgalactotigonin was successfully separated (Figs. 1b, c). Each mixture of saponins which are large in molecular size and have the same number and kind of sugars[10] had not been resolved by the conventional paper chromatography except by the chromatography on formamide-impregnated paper.[10,13] Thus the thin layer chromatography offers a rapid, sensitive and convenient method also in the field of steroid saponin and, above all, it gains an advantage over paper chromatography in resolving power of closely related saponins of large molecular size.

Saponin peracetates and permethylates also gave, respectively, a distinct single spot on the thin layers (Rf values given in Table II) and the Rf values of peracetates were nearly inversely proportional to their acetyl group content. Application of this method to the analyses of mixtures of saponin peracetates and of acetylation products of some saponins afforded the chromatograms shown in Fig. 2. When acetylated once with acetic anhydride-pyridine at room temperature followed by crystallization the products from gracillin and timosaponin A-III gave, respectively, only one spot of the corresponding peracetate on the thin layers, while those from F-gitonin and tomatine gave two spots (Figs. 2c, d).*3 Since the latters had the OH absorption in their infrared spectra and by repeated acetylation they gave the products which had no more OH absorption and showed the faster moving spot of peracetate alone on the chromatograms, the slower moving substance was regarded as an incompletely acetylated derivative. It was also found (Fig. 2e) that peracetate of gracillin was partially deacetylated when passed through an alumina column. The present method may offer a simple and useful means to follow the courses of acetylation and methylation reactions of saponins.

*3 The product from dioscin also gave two spots and even by repeated acetylation the product showing one spot was not obtained. Dioscin acetate (m.p. 143~145°, $[\alpha]_D^{12}$ −46°)[14] which had been prepared by one acetylation followed by chromatography on Brockmann alumina using benzene as the solvent was found to have the OH absorption in the IR spectrum and to give a few spots on the thin layer. A study on the preparation and the properties of pure dioscin peracetate is in progress.

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14) T. Tsukamoto, T. Kawasaki, A. Naraki, T. Yamauchi: Yakugaku Zasshi, 74, 984 (1954).

Table I.

	Saponin	Composition (Number of OH Group)[a]	Rf[b] A	B	C	D	Color Change[c] of Spot
1	Trillin[15]	dios+glc (4)	1.00	1.00	1.00	1.00	R P→Y G
2	Yononin[16]	yono+ara (4)	0.97	1.08	1.07		B P→D P
3	Timosaponin A-I[17]	sarsa+gal (4)	0.85	0.86	1.03		B→Y B
4	Tokoronin[16]	tokoro+ara (5)	0.77	0.89	0.87	0.98	B→D B
5	Prosapogenin A of Dioscin[15]	dios+glc+rha (6)	0.68	0.72	0.55		R P→Y G
6	Timosaponin A-III[17]	sarsa+gal+glc (7)	0.52	0.67	0.46	0.84	B→Y B
7	Dioscin[15,20]	dios+glc+2rha (8)	0.46	0.61		0.77	R P→Y G
8	Gracillin[18,20]	dios+2glc+rha (9)	0.45	0.58		0.76	R P→Y G
9	Desgalactotigonin[d]	tigo+gal+2glc+xyl (12)	0.40	0.55		0.71	B P→D B
10	F-Gitonin[e]	gito+gal+2glc+xyl (13)	0.33	0.50		0.67	P→D P
11	Gitonin[f]	gito+2gal+glc+xyl (13)	0.34	0.54			P→D P
12	Desglucodigitonin[g]	digito+2gal+glc+xyl (14)	0.30	0.51			Y B→B
13	Kikubasaponin[19,20]	dios+3glc+rha (12)	0.19	0.33		0.49	R P→Y G
14	Tomatine	tomat+gal+2glc+xyl (12+1NH)	0.25	0.44		0.60	DBl→DBl

a) dios : diosgenin yono : yonogenin sarsa : sarsasapogenin tokoro : tokorogenin tigo : tigogenin digito : digitogenin gito : gitogenin tomat : tomatidine glc : D-glucose ara : L-arabinose gal : D-galactose rha : L-rhamnose xyl : D-xylose

b) Rf values are represented in the relative values to those of trillin.*6

Developing solvent (Developing time, hr.)
- A : BuOH saturated with H_2O (10)
- B : $CHCl_3$-MeOH-H_2O (65:35:10) (lower phase) (1)
- C : $CHCl_3$-MeOH (80:20) (0.5)
- D : BuOH-AcOH-H_2O (4:1:5) (upper phase) (3)

c) When sprayed with 10% H_2SO_4 followed by heating at about 150° and then left stand at room temp.
R : red P : purple Y : yellow G : green B : brown D : dark Bl : blue

d) m.p. 284~286° (decomp.), $[\alpha]_D^{25}$ −100° (c=0.15, pyridine)[10]

e) m.p. 252~255° (decomp.), $[\alpha]_D^{25}$ −58.5° (c=0.53, pyridine)[10]

f) m.p. 238~241° (decomp.), $[\alpha]_D^{20}$ −60.0° (c=0.2, pyridine)[10]

g) m.p. 243~245° (decomp.), $[\alpha]_D^{20}$ −71.4° (c=0.28, pyridine)[10]

Table II.

	Saponin derivative	Number of acetoxyl or methoxyl group	Rf value[a]
1.	Trillin tetraacetate[15]	4	1.00
2.	Timosaponin A-I tetraacetate[17]	4	0.96
3.	Hexaacetate of prosapogenin A of dioscin[15]	6	0.75
4.	Timosaponin A-III heptaacetate[b]	7	0.69
5.	Heptaacetate of prosapogenin A of gracillin[18]	7	0.53
6.	Gracillin nonaacetate[19]	9	0.50
7.	Kikubasaponin dodecaacetate[19]	12	0.19
8.	F-Gitonin tridecaacetate[c]	13	0.25
9.	Tomatine tridecaacetate[d]	13	0.23
10.	Timosaponin A-III heptamethylate[17]	7	0.34
11.	Dioscin octamethylate[20]	8	0.36
12.	Gracillin nonamethylate[20]	9	0.46
13.	F-Gitonin tridecamethylate[e]	13	0.14

a) Rf values of acetates (1~9) are represented in the relative values to that of trillin tetraacetate.*6
Developing solvent (Developing time) :
For acetates (1~9) : $CHCl_3$-EtOH (100:2) (0.5 hr.)
For methylates (10~13) : benzene-Me_2CO (80:20) (0.5 hr.)

b) m.p. 123~126°, $[\alpha]_D^{15}$ −93.2° (c=2.41, $CHCl_3$), to be published.

c) m.p. 131~138°, prepared by repeated acetylation with Ac_2O-pyridine, to be published.

d) m.p. 134~142°, prepared as above, to be published.

e) a syrup, to be published.

15) T. Tsukamoto, T. Kawasaki, T. Yamauchi : This Bulletin, **4**, 35 (1956).
16) T. Kawasaki, T. Yamauchi : Yakugaku Zasshi, **83**, 759 (1963).
17) *Idem* : This Bulletin, **11**, 1221 (1963).

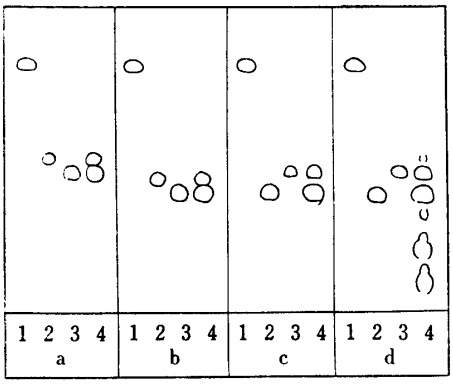

Fig. 1. Separation of Saponins on Silica Gel-Gypsum
(Solvent system : $CHCl_3$–MeOH–H_2O (65:35:10))

a : 1. Trillin
2. Dioscin
3. Gracillin
4. Dioscin+gracillin

b : 1. Trillin
2. Gitonin
3. Desglucodigitonin
4. Gitonin+desglucodigitonin

c : 1. Trillin
2. F-Gitonin
3. Desgalactotigonin
4. F-Gitonin+desgalactotigonin

d : 1. Trillin
2. F-Gitonin
3. Desgalactotigonin
4. *Dig. purp.* leaves saponins

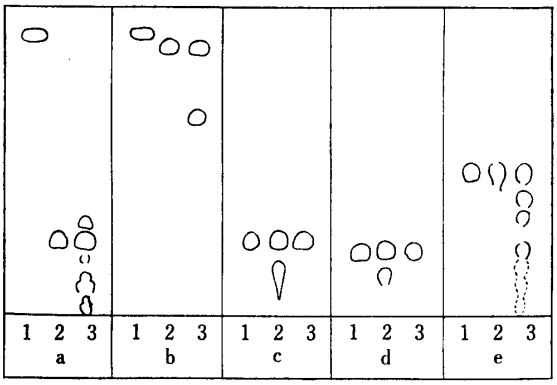

Fig. 2. Separation of Saponin Acetates on Silica Gel-Gypsum
(Solvent system : $CHCl_3$–EtOH (100:2))

a : 1. Trillin tetraacetate
2. F-Gitonin tridecaacetate
3. Acetates of *Dig. purp.* leaves saponins

b : 1. Trillin tetraacetate
2. Timosaponin A-I tetraacetate
3. Timosaponin A-I tetraacetate+timosaponin A-III heptaacetate

c : 1. F-Gitonin tridecaacetate
2. Acetylation (once) product of F-gitonin with Ac_2O–pyridine
3. Acetylation (twice or three times) product with Ac_2O–pyridine

d : 1. Tomatine tridecaacetate
2. Acetylation (once) product of tomatine with Ac_2O–pyridine
3. Acetylation (twice or three times) product with Ac_2O–pyridine

e : 1. Gracillin nonaacetate
2. Chromatography of gracillin nonaacetate (0.1 g.) on Brockmann Al_2O_3 (3 g.) benzene fraction
3. Same as 2, $CHCl_3$ fraction

Experimental

Preparation of Chromatoplates——Glass plates (20×5×0.3 cm.) were coated with a layer of silica gel containing gypsum*4 at a thickness of 250 μ with the aid of a commercial applicator (Yazawa, Model HCG-100). The coating mixture was prepared by adding 15 g. of silica gel-gypsum to 43 ml. of H_2O with vigorous shaking. After coating the plates were dried at room temperature and then activated by heating in an electric oven at 100° for 1 hr.

Compounds Examined——All standard saponins and their derivatives were those which had been isolated or prepared in our laboratory (refer to the literatures cited in Tables I and II) . The sample of tomatine (m.p. 270~271° (decomp.), $[\alpha]_D^{31}$ −31° (c=1.62, pyridine)) was kindly donated by Dr. Sato.

Application of Samples and Development——Samples were dissolved at 1~2% w/v concentration in $CHCl_3$–MeOH (1:1) mixture (for saponin) or in $CHCl_3$ (for derivatives) and applied in a line 3~4 cm. from the bottom edge of the plate in 1~2 μl. amounts using a capillary. Development was made by the

*4 Commercial silica gel (Kanto Chemical Co. Inc., 100~200 mesh, for chromatographic use) was ground in a mortar and mixed with 1/14 part of gypsum according to the suggestion of Dr. Furuya. "Silica Gel G" (Merck) and the silica gel with gypsum prepared by Dr. Furuya gave the similar results.

18) T. Tsukamoto, T. Kawasaki : This Bulletin, **4**, 104 (1956).
19) T. Kawasaki, T. Yamauchi, R. Yamauchi : *Ibid.*, **10**, 698 (1962).
20) *Idem* : *Ibid.*, **10**, 703 (1962).

ascending method for a distance of about 12~14 cm. in a vessel closed with a glass plate, lined with filter paper for good solvent equilibration of the vessel and containing a solvent to a depth of 1~2 cm. The solvent systems used and the respective time of development are shown in Table I.

Detection——After the development the plates were air-dried, sprayed with 10% H_2SO_4, then heated at about 150° for 5 min., and left stand at room temperature. The final color of a spot (Table I) was almost analogous to that of the corresponding aglycone with conc. H_2SO_4 reported by Hara.[5b] The limit of detection was less than 1 γ*[5] under the day light.

Because Rf values varied depending on the activity of the chromatoplate, the samples (except for the methylates) were run in parallel with trillin and dioscin, or trillin tetraacetate and gracillin nonaacetate and their Rf values (Tables I and II) were represented in the relative values to those*[6] of trillin and trillin tetraacetate.

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Summary

Thin layer chromatography on silica gel-gypsum was applied to steroid saponins and their derivatives such as peracetates and permethylates. The method has many advantages over paper chromatography in speed, sensitivity, and, above all, in resolving power of the closely related high molecular weight saponins and of the slightly polar derivatives.

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*[5] 0.1 γ of trillin was detected.

*[6] The Rf value of trillin in respective solvent system was about 0.45 (BuOH saturated with H_2O), 0.82 ($CHCl_3$-MeOH-H_2O (65:35:10)), 0.63 ($CHCl_3$-MeOH (80:20)) and 0.78 (BuOH-AcOH-H_2O (4:1:5)). Trillintetraacetate had the Rf value of about 0.92 in $CHCl_3$-EtOH (100:2) system.