166—167.5°). IR ν_{max}^{KBr} cm^{-1}: 1720 (CO). NMR ($CDCl_3$) δ: 3.75 (6H, s, $-OCH_3$), 7.5 (10H, s, arom.). Mass Spectrum *m/e*: M^+, 352. *Anal.* Calcd. for $C_{20}H_{16}O_4S$: C, 68.16; H, 4.58. Found: C, 67.95; H, 4.39.

Dimethyl 2-(*p*-Anisyl)-5-phenylthiophene-3,4-dicarboxylate (VIb)——To a mixture of IIe (302 mg) and dimethyl acetylenedicarboxylate (142 mg) in benzene (2 ml) was added a solution of trifluoroacetic anhydride (210 mg) in benzene (1 ml) at room temperature. The mixture was stirred for 5 hr, and then worked up as above to give 350 mg (90%) of VIb, pale yellow oil, which crystallized on standing. mp 101—108° (lit.[4b] mp 110.5—111.5°). Distillation gave the analytical sample, bp 160—165° (1×10^{-4} mmHg, bath temp.), colorless oil. Mass Spectrum *m/e*: M^+, 382. *Anal.* Calcd. for $C_{21}H_{18}O_5S$: C, 65.95; H, 4.74. Found: C, 65.98; H, 4.83.

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Fluorescence Densitometric Method for the Simultaneous Determination of Siomycins using Silicagel-Sintered Plate

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A simple and rapid fluorodensitometric method for simultaneous determinations of siomycin A, siomycin B, and siomycin D_1 in siomycins was established.

The method is based on the fluorescence reaction of siomycins with sulfuric acid after separation on thin-layer chromatographic plate, and can be applied satisfactorily to simultaneous determinations in the concentration range of 5—1000 ng per spot.

Keywords——siomycins; fluorodensitometry; simultaneous determination; silicagel-sintered plate; thin-layer chromatography

Individual fluorodensitometric methods for determinations of siomycin A (A), siomycin B (B), and siomycin D_1 (D_1) have been reported previously.[2] However, the methods for A and D_1 require a troublesome, time-consuming procedure such as two-dimensional development for separation. By examining the quantitative conditions in more detail, we established a simple and rapid method for simultaneous determinations of A, B, and D_1 with one-dimensional development using a Yamato Replate-100 (Yamato Scientific Co., Ltd., Nihonbashi Hon-cho, Chuo-ku, Tokyo). This improved method is more sensitive and simple than previous methods.[2]

Experimental

Apparatus——A Hitachi MPF-2A spectrofluorometer equipped with a Hitachi thin-layer chromatogram (TLC) scanning attachment was used to measure the fluorescence of the spot on the plate. The fluorescence intensity was recorded on a Hitachi J 301 recorder and simultaneously integrated with a Hitachi J 201 integrator. We also used a Hamilton dosing syringe (10 μl), a rectangular developing chamber ($9 \times 22 \times 22$ cm), a Lapine hot plate (775 watts, 115 volts), a Toshiba Type F1-3L ultraviolet lamp, and pretreated Yamato Replate-100.

Pretreatment of Yamato Replate-100——Commercially available plate (0.2 mm, 10×20 cm) was developed with a mixture of chloroform and methanol (95: 5, v/v) for 50 min in the developing chamber saturated

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with solvent vapor by the ascending method. The plate was dried for 5 min at 105°, then kept in air at room temperature for 30 min.

Reagents——Standard Siomycin A, B, and D_1: These were obtained as reported previously.[2)]

Standard Solution of A: Fifty milligrams of standard siomycin A was dissolved in chloroform to make 500 ml of stock solution. This solution was stable for a month when stored in a refrigerator. Standard solution were prepared by diluting the stock solution with chloroform to the concentrations required before use.

Developing Solvent: A mixture of chloroform and methanol (95: 5, v/v) was used.

Chloroform, methanol, and sulfuric acid were reagent grade.

Standard Procedure for A (B or D_1)——Ten microliters of sample solution containing less than 1 μg of A (B or D_1) was spotted on the Yamato Replate-100 and developed with a mixture of chloroform and methanol (95: 5) for 50 min in the developing chamber saturated with solvent vapor. After the plate had been dried for 10 min in air at room temperature, it was sprayed with 15N sulfuric acid, then heated for 20 min at 105±2° on a hot plate. Within 80 min, the position of the fluorescence spot formed by A (B or D_1) was detected with an ultraviolet lamp (365 nm), and then the fluorescence intensity of the spot at 452 nm at the excitation wavelength, 398 nm, was measured by the peak-height method by setting the slit (1×6, mm) of the TLC scanning attachment at the center of the spot at right angles to the direction of the solvent flow.

The calibration curve was obtained by plotting the peak-height values *versus* the concentrations of the standard solutions of A on the same plate as described above. The concentration of A (B or D_1) in the sample solution was then obtained by calculation.

Results and Discussion

Siomycin B was readily separated from A and D_1 under our experimental conditions. Therefore, the results are discussed with respect to the separation of A and D_1.

Pretreatment of TLC Plate

The examination was carried out using ten plates and 10 μl of mixed solution containing 1 μg of A, B, and D_1.

1) **Effect of Predevelopment**——Table I shows the effect on the separation of A, B, and D_1. The distance between the spots of A and D_1 was longer than that with no predevelopment by which they were not separated sufficiently, showing that it aided separation.

Table I. Effect of Predevelopment on the Separation of Siomycin A,B and D_1 on TLC Plate

Method	*Rf*		
	A	B	D_1
Predevelopment	0.34—0.42	0.47—0.56	0.22—0.32
No predevelopment	0.29—0.39	0.42—0.54	0.20—0.28

2) **Activation Conditions of the Plate after Predevelopment**——Table II shows the activation conditions of a Yamato Replate-100 for separation of A, B, and D_1. Heating the plate at 105±2° for 3 or 5 min after predevelopment, lengthened the distance between the spots of A and D_1. The most effective separation was obtained with 5 min heating.

Table II. Activation Conditions of Yamato Replate-100 after Predevelopment

Temperature (°C)	Time (min)	*Rf*		
		A	B	D_1
24±2	30	0.34—0.36	0.48—0.49	0.25—0.26
105±2	3	0.50—0.51	0.58—0.59	0.33—0.34
105±2	5	0.40—0.41	0.54—0.56	0.29—0.30
105±2	10	0.33—0.34	0.46—0.47	0.25—0.26
105±2	15	0.37—0.38	0.50—0.51	0.27—0.28

3) **Effect of Standing Time**——The effect on separation of A, B, and D_1 was examined in air at room temperature over time range of 30 to 70 min after heating. As shown in Table III, no difference in the distance between the spots of A and D_1 appeared. We selected the standing time of 30 min by considering the rapidity desired for the determination.

Table III. Effect of Standing Time on the Separation of Siomycin A, B and D_1 on TLC Plate

Time (min)	*Rf*		
	A	B	D_1
30	0.36—0.37	0.47—0.49	0.27—0.29
50	0.31—0.32	0.42—0.43	0.23—0.24
70	0.37—0.38	0.50—0.51	0.27—0.28

Selection of Developing Solvent

Four combinations of the chloroform-methanol solvent system were examined. As shown in Table IV, the maximum distance between the spots of A and D_1 was obtained using 93: 7 chloroform–methanol, but a small tailing which caused error was found below the spots. On the other hand, although 95: 5 was somewhat inferior to the 93: 7 mixture, it did not cause tailing. Thus, 95: 5 chloroform–methanol was selected as the suitable solvent for the development.

Table IV. Effect of Solvent on the Separation of Siomycin A, B and D_1 on TLC Plate

Solvent system (chloroform : methanol)	*Rf*		
	A	B	D_1
97 : 3	0.13—0.14	0.20—0.21	0.06—0.07
96 : 4	0.21—0.23	0.30—0.31	0.14—0.15
95 : 5	0.37—0.40	0.49—0.51	0.29—0.30
93 : 7	0.59—0.61	0.75—0.76	0.49—0.50

Fluorescence Spectral Characteristics of Spots Formed by A, B, and D_1

Table V shows the uncorrected excitation and emission spectra of the fluorescent spots formed by A, B, and D_1 on TLC plate. The maximum wavelengths of excitation and emission spectra coincided well respectively, and hence the fluorescing compounds in the spots were presumed to be the same. The excitation maximum of 398 nm and the emission maximum of 452 nm were selected for measurement of the fluorescence intensities of the spots.

Table V. Fluorescence Spectral Characteristics of the Fluorescent Products Formed by Siomycin A, B and D_1 on TLC Plate

Siomycin	Peak wavelengths (nm)	
	Excitation maxima	Emission maxima[a]
A	398	433, *452*
B	398	432, *452*
D_1	398	434, *451*

a) Wavelengths of the main peak are italicized.

Fluorescence Stability

The fluorescence stability of the spot formed by 1 μg of A was examined under room light and room temperature. As shown in Fig. 1, after fluorescence development, the fluorescence was stable for 80 min.

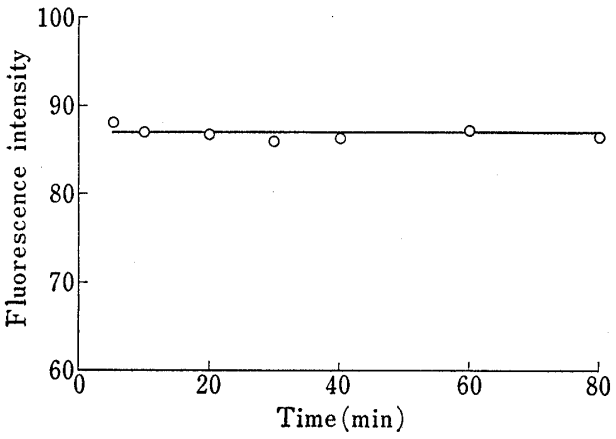

Fig. 1. Stability of Fluorescent Product Formed by Siomycin A on TLC Plate

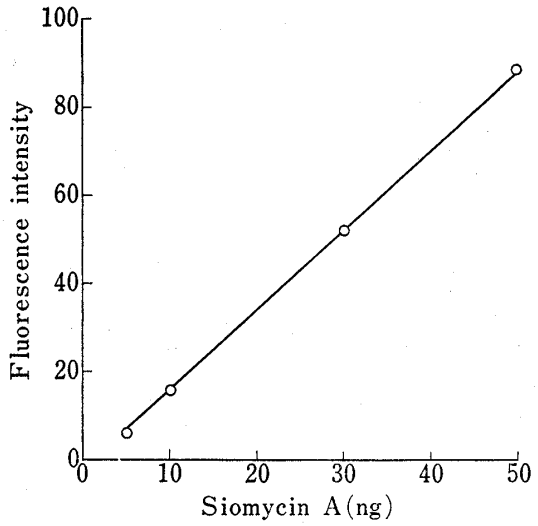

Fig. 2. Calibration Curve for Siomycin A

Application of the Calibration Curve Obtained from A to Determinations of B and D_1

In the range of 0.2—2.0 μg, the fluorescence intensities of the spots formed by A, B and D_1 were measured by both the peak-height and integration methods, and then the values were used to test whether the calibration curve for A could be applied to the determinations of B and D_1.

The calculated results for both values by covariance analysis indicated that the calibration curve obtained with peak-height values could be applied to the determination of B and D_1, but that obtained with integration values could not.

Thus, the calibration curve for A of peak-height values could be simultaneously applied to the determinations of B and D_1.

Calibration Curve

Fig. 2 shows the calibration curve for A in the concentration range of 5—50 ng per spot. A linear relationship was observed between the fluorescence intensity and the concentration with a 1.4% coefficient of variation on a TLC plate.

Regression Analysis

The standard procedure was applied to the determination of A in the concentration range of 5—41 ng in ten mixed sample solutions containing 27—91% of B and D_1 by weight. As shown in Table VI, the calculated results of regression analysis between the theoretical (x) and experimental values (y) indicated that the procedure gave the corrected values for the determination of A with a 6% coefficient of variation.

Also, as for A, the procedure was applied to the determination of B and D_1 in the concentration ranges of 5—40 ng and 5—50 ng, respectively, in mixed samples consisting of A, B, and D_1. It was confirmed that the procedure gave the correct values for B and D_1 with 2 and 9% coefficients of variation respectively.

The results described above show that the standard procedure could be applied to the simultaneous determinations of A, B, and D_1 on a TLC plate.

Table VI. Regression Analysis for Assay of Siomycin A in the Presence of Siomycin B and D_1[a]

Sample No.	Components in 10 μl of mixed sample solution (ng)			Found (ng)
	A(x)	B	D_1	A(y)
1	5	5	50	7
2	5	5	50	7
3	10	5	40	11
4	10	5	40	12
5	21	5	30	21
6	21	5	30	23
7	31	5	20	32
8	31	5	20	33
9	41	5	10	38
10	41	5	10	42

a) regression equation: $y=0.935x+2.40$, s=1.36, c.v.=6.00

Advantages of This Method

This method is 6—40 times more sensitive than previous method,[2] and is also simpler.

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Biosynthesis of Streptothricin Antibiotics. III.[1] Incorporation of D-Glucosamine into D-Gulosamine Moiety of Racemomycin-A

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When D-glucosamine-1-^{14}C was added to a growing culture of *Streptomyces lavendulae* ISP-5069, a high incorporation of radioactivity into racemomycin-A was observed. Radioactivity was exclusively located in D-gulosamine moiety of the molecule.

Keywords——antibiotic biosynthesis; *Streptomyces lavendulae* ISP-5069; incorporation of D-glucosamine-1-^{14}C; D-glucose-U-^{14}C; D-glycerol-U-^{14}C; radioactive racemomycin-A; direct precursor; radioactivity scanning; cellulose partition chromatography

As reported in the previous paper,[3] D-glucose-U-^{14}C, which was added at the early production stage of racemomycin-A by *Streptomyces lavendulae* ISP-5069, was incorporated into D-gulosamine moiety in racemomycin-A. Further studies on the biosynthesis of aminosugar moiety in racemomycin-A molecule made it clear that D-glucosamine was incorporated into

1) Y. Sawada, H. Sakamoto, T. Kubo, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **24**, 2480 (1976).
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3) Y. Sawada, T. Kubo, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **24**, 2163 (1976).