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Note

Thin-layer chromatographic analysis of the OA-6129 group of carbapenem antibiotics in fermentation broths

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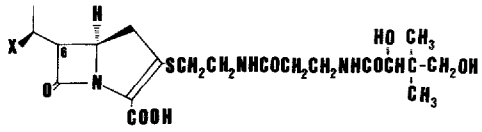
Okabe *et al.*[1] previously described a silica gel thin-layer chromatographic (TLC) method for analysis of PS-5, a carbapenem antibiotic. The method involved solvent extraction from broth filtrate, spotting and development on a silica gel TLC plate, treatment with the Ehrlich reagent and densitometric quantitation. When a new series of carbapenems designated the OA-6129 group were discovered in fermentation broth from *Streptomyces* sp. OA-6129[2–4], a new TLC system was required for quantitative and qualitative determination of the carbapenems. The method of Okabe *et al.* was devised specifically for PS-5 which was the least hydrophilic of the known carbapenem compounds, so a more general method was developed which was applicable to hydrophilic carbapenem analogues such as epithienamycins[5], olivanate[6] and the OA-6129 group.

This communication reports an improved silica gel TLC method which is useful for quantitative and qualitative analysis of most carbapenem compounds including the OA-6129 group.

MATERIALS AND METHODS

Antibiotics

OA-6129A, OA-6129B_2 and OA-6129C were produced as the sodium salts, as described previously[2]. The reported ε values of OA-6129B_2 and OA-6129C, 5400 and 7600 respectively, have been revised to 8400 and 10300 by repeated column chromatography[2].



	X	C-C
OA-6129A	H	R
OA-6129B_1	OH	R
OA-6129B_2	OH	S
OA-6129C	OSO_3H	R

Fig. 1. Structures of the OA-6129 group of carbapenem compounds.

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Fermentation

Streptomyces sp. OA-6129 produces the OA-6129 group of carbapenem compounds presented in Fig. 1[2].

As reference carbapenem-producing microorganisms, *Streptomyces cattleya* (thienamycin)[7] and *Streptomyces fulvoviridis* A933 (PS-5, PS-6, PS-7, PS-8, epithienamycins A, B, C and D, MM-13902 and MM-17880)[8-11] were employed and were fermented as described previously.

Spectrophotometric measurement of the coloured products of OA-6129B$_2$ and OA-6129C

One millilitre of an aqueous solution of OA-6129B$_2$ or OA-6129C (500 μg/ml) was mixed with 1 ml of the Ehrlich reagent solution and heated in an oven at 105°C for 5 min. The coloured solution was diluted in methanol and cooled to room temperature for 30 min. The UV–visible spectrum of the dilute solution was measured in a Hitachi 200-20 UV–visible spectrophotometer using methanol as reference.

Heat treatment of fermentation broth filtrate

An about 90-h fermentation broth of *Streptomyces* sp. OA-6129 was centrifuged. The supernatant solution was acidified to pH 1.5–2.0 with 2 *M* hydrochloric acid, and heated for 5 min in a bath of boiling water so that carbapenem compounds in the supernatant were completely decomposed. The heat-treated solution was passed through filter-paper and the filtrate was adjusted to pH 8.0 with 2 *M* sodium hydroxide. A known quantity of an authentic carbapenem compound was dissolved in the heat-treated filtrate to give a reference concentration.

When a carbapenem spot on a silica gel TLC plate was found to have a crescent shape, 6% sodium chloride was added to the heat-treated or intact broth filtrate at a mixing ratio of 1:1, yielding more spherical carbapenem spots.

Silica gel TLC

Five microlitres of an intact, heat-treated or sodium chloride-containing broth filtrate were applied on a pre-coated silica gel TLC plate (Article No. 5715; E. Merck, Darmstadt) and then dried *in vacuo* at room temperature for 20 min. The TLC plate was developed at 5–10°C in chloroform–methanol–0.01 *M* phosphate buffer, pH 7.5 (8:6:1) or as indicated. After the developing solvent had been evaporated with cold air, the chromatogram was dipped in an Ehrlich reagent bath containing 300 mg of *p*-dimethylaminobenzaldehyde in a mixture of 54 ml *n*-butanol, 9 ml ethanol and 9 ml concentrated hydrochloric acid. It was then heated in an oven at 105°C for 3 min.

Densitometry

The colour intensity of a carbapenem spot on the chromatogram was measured about 30 min after visualization in a Shimadzu CS-920 High Speed Thin Layer Chromatographic Scanner at a wavelength of 575 nm.

Bioautography

A 32 × 24 cm rectangular glass tray was filled with 100 ml of molten agar medium inoculated with *Comamonas terrigena* B-996. A silica gel TLC plate carrying carbapenem compounds was contacted with the surface of the seeded agar medium

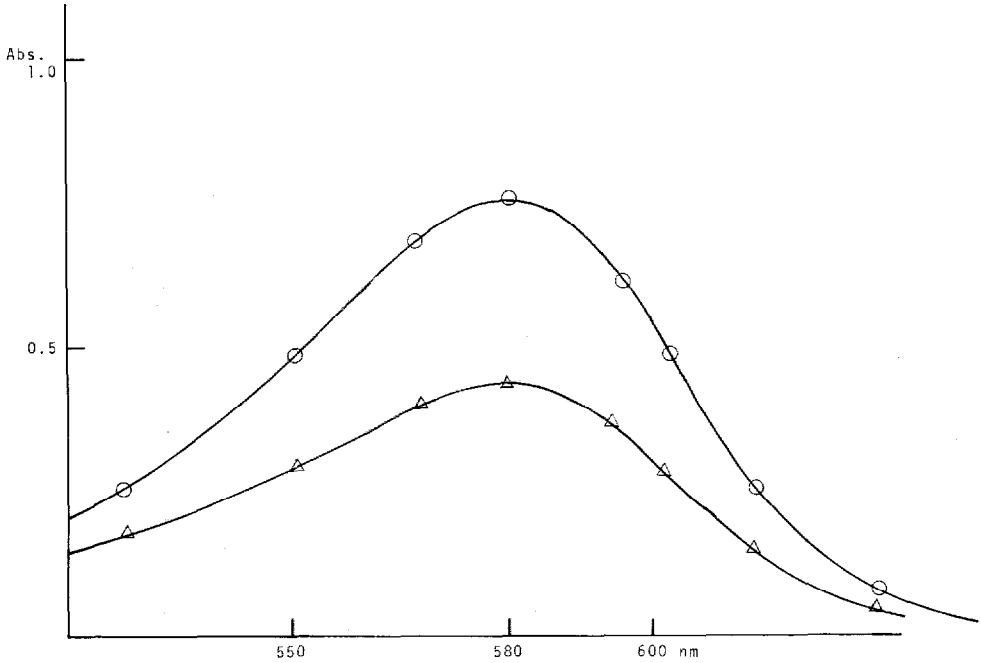

Fig. 2. Visible spectra of the Ehrlich colouration products of OA-6129B_2 and OA-6129C. ○—○, OA-6129B_2 (20 μg/ml); △—△, OA-6129C (10 μg/ml).

for 20 min. The chromatogram was removed and the assay tray was incubated at 30°C for 20 h.

RESULTS AND DISCUSSION

UV–visible spectra of the coloured products of OA-6129B_2 and OA-6129C

The reaction products of OA-6129B_2 and OA-6129C with the Ehrlich reagent show a visible absorption maximum at 580 nm (Fig. 2). When broth filtrates were

TABLE I

R_F VALUES OF OA-6129A, OA-6129B_2, OA-6129C AND PS-5 ON A SILICA GEL TLC PLATE

Solvent systems: 1 = chloroform–methanol–0.01 *M* phosphate, pH 7.5 (8:6:1); 2 = chloroform–methanol–water (8:6:1); 3 = *n*-butanol–ethanol–chloroform–water (5:5:1:2); 4 = *n*-butanol–methanol–chloroform–water (5:5:2:1); 5 = acetonitrile–water (3:1); 6 = water-saturated *n*-butanol.

Solvent system	*0.01 M Phosphate buffer, pH 7.5*				*Broth filtrate*		
	PS-5	*OA-6129A*	*OA-6129B_2*	*OA-6129C*	*OA-6129A*	*OA-6129B_2*	*OA-6129C*
1	0.46	0.42	0.35	0.35	0.41	0.28	0.20
2	0.46	0.42	0.35	0.35	0.38	0.26	0.21
3	0.39	0.43	0.31	0.41	0.35	0.20	0.18
4	0.55	0.54	0.50	0.58	0.55	0.35	0.29
5	0.38	0.37	0.25	0.37	0.33	0.24	0.19
6	0.18	0.12	0.07	0.12			

analysed by TLC, the quantitation of carbapenem compounds was found to be more suitable at 575 nm than at 580 nm, probably because of the impurities present in broth filtrates.

R_F data of the authentic carbapenem compounds dissolved in buffer and the heat-treated broth filtrate

Table I lists the R_F values of OA-6129A, OA-6129B_2 and OA-6129C dissolved in 0.01 *M* phosphate buffer, pH 7.5, and the heat-treated broth filtrate. OA-6129B_2 and OA-6129C in the buffer give the same or similar R_F values, but in the heat-treated broth filtrate they are clearly separable presumably because of the protein impurities. In all the systems, OA-6129A produced round spots, whereas OA-6129B_2 and OA-6129C resulted in crescent shaped spots.

Treatment of the OA-6129B_2 and OA-6129C spots

In principle, a round spot is more suitable for densitometry than a crescent shaped spot, so analytical conditions were sought which would result in a round spot of carbapenem on a silica gel TLC plate. It was found during purification steps that the presence of a high concentration of a salt in column fractions resulted in round

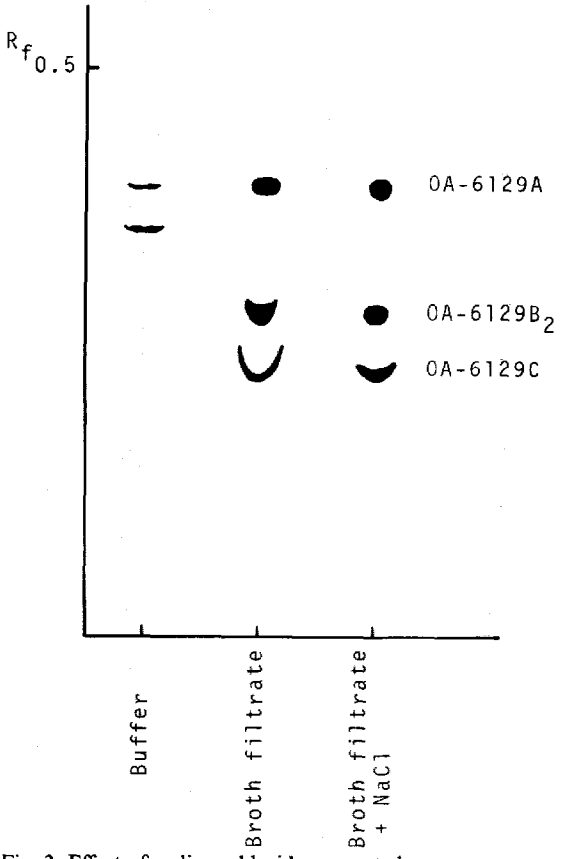

Fig. 3. Effect of sodium chloride on spot shape.

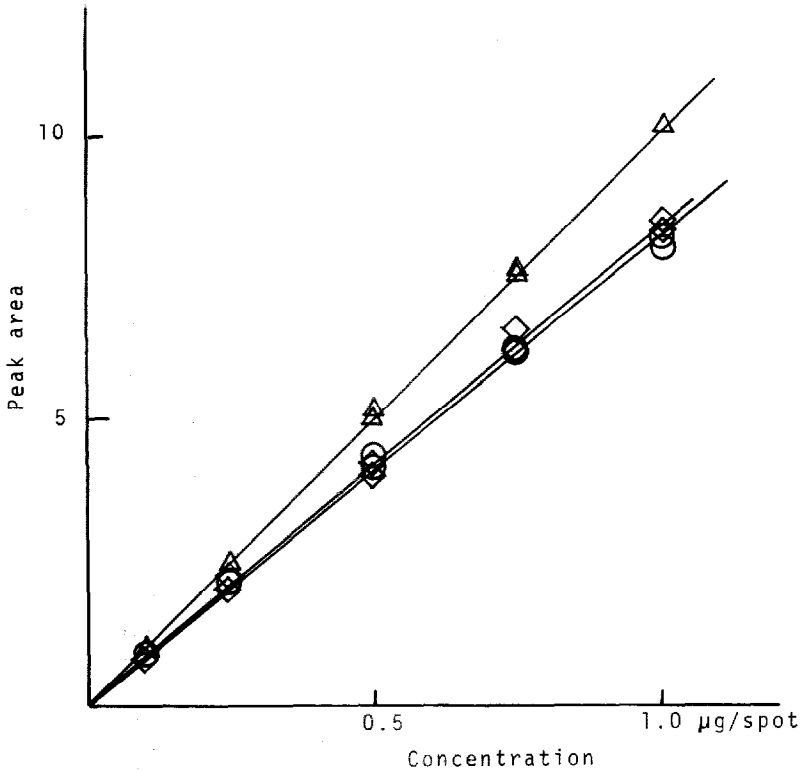

Fig. 4. Calibration curves for TLC quantitation of OA-6129A (◇), OA-6129B_2 (○) and OA-6129C (△).

spots of carbapenems on a silica gel TLC plate. The effect of sodium chloride on OA-6129B_2 and OA-6129C was examined in detail, revealing an optimum concentration in the heat-treated broth filtrate of about 3% for OA-6129B_2. OA-6129C still had a crescent shape even in the presence of high concentrations of sodium chloride. The effect of sodium chloride is illustrated in Fig. 3 for the broth filtrate of *Streptomyces* sp. OA-6129 containing OA-6129A, OA-6129B_2 and OA-6129C.

Determination of OA-6129A, OA-6129B_2 and OA-6129C in fermentation broths

Known quantities of OA-6129A, OA-6129B_2 and OA-6129C were dissolved in the heat-treated broth filtrate containing 3% of sodium chloride and analysed under the specified conditions. Fig. 4 shows that the amounts of the carbapenems are linearly related with the densitometric peak areas in the concentration range of 0.1–1.0 μg per spot. The correlation coefficients are 0.9994, 0.9990 and 0.9999 for OA-6129A, OA-6129B_2 and OA-6129C, respectively. The satisfactory linear ranges of the three carbapenems extend to about 5 μg per spot; above this their calibration curves become non-linear. Although the very small amount of OA-6129B_1 available prevented a detailed comparison with the other three analogues, a highly purified preparation of OA-6129B_1 could be separated from OA-6129B_2 in the absence of salts on a silica gel TLC plate using the described system.

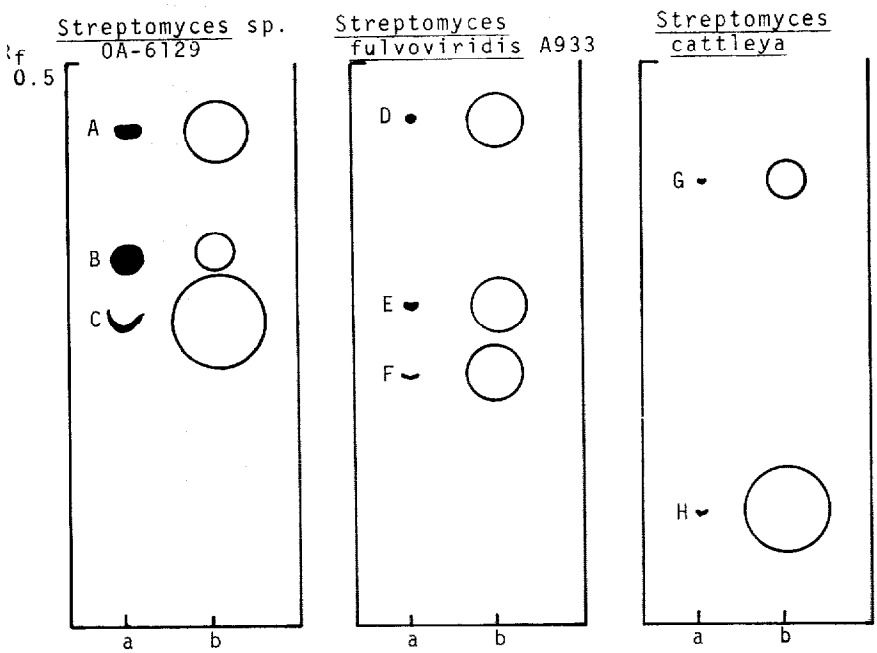

Fig. 5. Comparison of the Ehrlich colouration (a) with bioautography (b). A = OA-6129A; B = OA-5129B$_1$ and B$_2$; C = OA-6129C; D = PS-5; E = epithienamycins A and C; F = MM 17880 and MM 13902; G = N-acetylthienamycin; H = thienamycin.

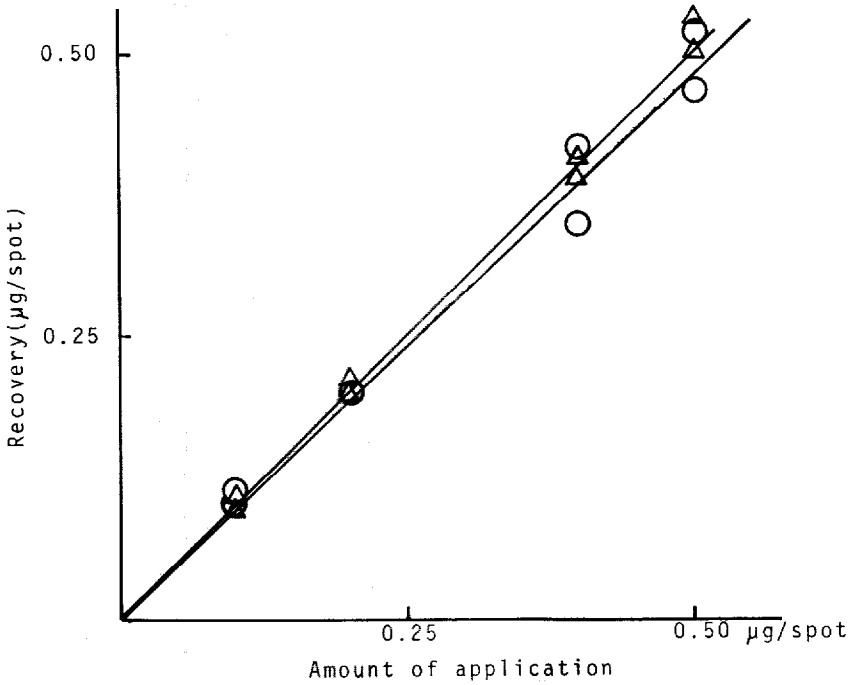

Fig. 6. Recovery of OA-6129B$_2$ (○) and OA-6129C (△) in the silica gel TLC assay.

Comparison of densitometry with bioautographic analysis

As carbapenem analogues differ greatly in antimicrobial potency, it is possible that trace amounts of a new analogue will be undetected because of insufficient sensitivity of the Ehrlich reagent. Fig. 5 compares densitometric and bioautographic analyses. As expected, OA-6129B_2, which has the least antimicrobial activity among the three carbapenems, yields the smallest halo despite the largest spot area.

Recovery of OA-6129B_2 and OA-6129C from broth filtrate of Streptomyces sp. OA-6129

Known amounts of OA-6129B_2 and OA-6129C were added to fermentation broth of *Streptomyces* sp. OA-6129 and analysed as described. Fig. 6 demonstrates the very satisfactory recoveries of OA-6129B_2 and OA-6129C, indicating that the present TLC method is highly quantitative.

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