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THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF CARBAPENEM ANTIBIOTICS IN FERMENTATION BROTHS

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SUMMARY

A one-dimensional silica gel thin-layer chromatographic method has been devised for qualitative and quantitative analysis of carbapenem antibiotics in fermentation broths. The antibiotics were extracted from the broth filtrate with a solvent mixture of 1-butanol and chloroform (1:1) and developed on a silica gel thin-layer plate with a mobile phase of acetonitrile–0.75% acetic acid (9:2). Carbapenem compounds on the thin-layer plate were visualized as reddish pink spots with the Ehrlich reagent and quantitated by densitometry.

INTRODUCTION

Thin-layer chromatographic (TLC) methods have successfully been used for product analysis of fermentation broths of various antibiotics[1], but there is no satisfactory method for carbapenem compounds, as they are produced in small amounts in broths and are chemically very reactive. Usually this family of β-lactam antibiotics have been analysed by laborious methods such as paper chromatography, high voltage paper electrophoresis and high-performance liquid chromatography, mostly in combination with bioautography[2].

PS-5 is a carbapenem compound discovered in our laboratories which has excellent antimicrobial activity and is a β-lactamase inhibitor. As this β-lactam was originally produced in very small concentration by streptomycetes, extensive work has been carried to improve the fermentation and the strain. During these studies, a rapid and easy method was needed for qualitative and quantitative analysis of PS-5 and related carbapenem compounds in fermentation broths.

This communication describes a new silica gel TLC method for qualitative and quantitative analysis of carbapenem compounds in fermentation broths of streptomycetes. The method consists of solvent extraction of the antibiotics from fermentation broths, development on a silica gel TLC plate, visualization with the Ehrlich reagent and quantitation by optical densitometry.

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MATERIALS AND METHODS

Antibiotics

PS-5[2,3], PS-6[4], PS-7[4] and epithienamycins A, B, C and D[5] were produced as sodium salts in our laboratories. Aqueous solutions of the antibiotics were freshly prepared before use. Compound PS-5 was used as reference β-lactam throughout.

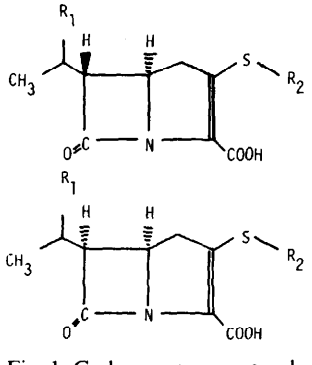
Fermentation

Streptomyces fulvoviridis A933[3] usually produced the carbapenem compounds presented in Fig. 1. Under our fermentation conditions where a supply of sulphur was limited, PS-5 was a main product, whereas the other carbapenems shown in Fig. 1 were minor components[4]. After fermentation, the broths were subjected to solvent extraction. With other carbapenem-producing streptomycetes such as *Streptomyces cremeus* subsp. *auratilis* and *Streptomyces argenteolus*, similar fermentation products were detected in broths by this TLC method.

Solvent extraction

For selection on an appropriate organic solvent and optimization of extraction conditions for carbapenem compounds, PS-5 in buffered solution was employed instead of fermentation broths. The aqueous PS-5 solution at a known concentration was quickly adjusted to the chosen pH with 0.4 *N* sulphuric acid and mixed with an organic solvent for about 1 min at room temperature on a vortex mixer. The organic and aqueous layers were immediately separated by centrifugation at 500 *g* for 5 min and then poured into large volumes of 0.01 *M* phosphate buffer, pH 8.0. The amounts of PS-5 in the organic and aqueous layers were measured by disk agar diffusion assay with *Comamonas terrigena* B996. The PS-5 extraction ability of a solvent is expressed in per cent extraction according to the following equation:

$$\text{Per cent extraction} = \frac{\text{solute in organic layer} \times 100}{\text{solute in original aqueous solution}}$$



No.	Compound	R_1	R_2
I	PS-5	H	$CH_2CH_2NHCOCH_3$
II	PS-6	CH_3	$CH_2CH_2NHCOCH_3$
III	PS-7	H	$CH{=}CHNHCOCH_3$
IV	Epithienamycin C	OH	$CH_2CH_2NHCOCH_3$
V	Epithienamycin D	OH	$CH{=}CHNHCOCH_3$
VI	Epithienamycin A	OH	$CH_2CH_2NHCOCH_3$
VII	Epithienamycin B	OH	$CH{=}CHNHCOCH_3$

Fig. 1. Carbapenem compounds.

TLC

Carbapenem compounds in organic solution were spotted on a pre-coated silica gel TLC plate (E. Merck, Darmstadt, G.F.R.) and developed with acetonitrile–0.75% acetic acid (9:2) 5–8°C. After removal of the developing solvent with a stream of by blowing the cold air, the chromatogram was dipped in the Ehrlich reagent and then heated in an oven at 100°C for 5 min. Carbapenem compounds appeared as reddish pink spots. The Ehrlich reagent consisted of 30 mg *p*-dimethylaminobenzaldehyde, 54 ml 1-butanol and 9 ml concentrated hydrochloric acid.

Disk agar diffusion assay

An overnight culture of *Comamonas terrigena* B996 on a nutrient agar slant medium was suspended in nutrient broth to give a seed cell suspension which had an optical density of 0.04 at 610 nm[3]. One per cent of the seed cell suspension was inoculated in molten agar medium consisting of 0.8% Kyokuto Nutrient Broth powder (Kyokuto Seiyaku Kogyo Co., Japan) and 1.0% Bacto-agar (Difco Laboratories, U.S.A.). Seven millilitres of the inoculated molten agar medium were poured into a 9-cm petri dish and allowed to solidify to provide a *Comamonas*-disk assay plate. The plate was incubated overnight at 28°C.

Densitometry

The colour intensity of a visualized carbapenem spot on a silica gel TLC plate was measured with a Shimadzu CS-910 High-Speed TLC Scanner (reading wavelength 555 nm; reference wavelength 700 nm; reflection mode; slit 1.25 mm in height and 1.25 mm in width; sensitivity × 10; zigzag scanning; stage scanning speeds 20 mm/min (abscissa), 20.4 mm/sec (ordinate); stage scanning strokes 170 mm (abscissa), 30 mm (ordinate); chart speed 20 mm/min).

RESULTS AND DISCUSSION

Extraction of PS-5 with water-immiscible organic solvents

Compound PS-5 is a weakly acidic substance, the pK_a of which has been estimated to be 3.46[6]. In penicillin production, compounds such as benzylpenicillin are effectively purified by extraction with organic solvents at an acidic pH followed by back-transfer into aqueous solutions at a neutral pH. Although it is theoretically possible to extract carbapenem compounds with organic solvents, the solvent extraction has hitherto not been incorporated into the purification procedure because these compounds are chemically reactive under acidic conditions. In view of the chemical structure of compound PS-5, we considered it worthwhile to test whether the antibiotic was extractable from acidic solutions with water-immiscible organic solvents. If the extraction step under acidic conditions could be completed in a short period of time, the loss of the carbapenem resulting from the acid treatment would be compensated by the efficiency of purification achieved. Even if the solvent extraction were not applicable on a large scale because of the length of time spent under acidic conditions, it would effectively serve as a preliminary purification and concentration step for TLC analysis of carbapenem compounds.

Table I shows the per cent extractions of compound PS-5 with various solvents, when the aqueous PS-5 solution (450 μg/ml) was extracted at pH 4.0 and room

TABLE I

EXTRACTION OF PS-5 WITH VARIOUS SOLVENTS

The aqueous PS-5 solution (450 μg/ml) was extracted at pH 4.0 and room temperature. The concentration of PS-5 both in organic layer and aqueous layer was measured by a disk agar diffusion assay with *Comamonas terrigena* B996.

Solvent	*Per cent extraction*
IPE*	2.1
MIBK**	4.3
n-Butyl acetate	1.2
Ethyl acetate	12.3
3-Methyl-1-butanol	54.3
1-Pentanol	60.0
2-Methyl-1-propanol	54.0
1-Butanol	51.0

* IPE = Diisopropyl ether.
** MIBK = Methyl isobutyl ketone.

temperature. Among the eight solvents, the butyl and pentyl alcohols yield an extraction yield of the antibiotic over 50%. Although 1-pentanol was found to be the best solvent, 1-butanol was used throughout this study because of its ready availability.

As a mixed solvent is often known to be more efficient in extraction than individual solvents, 1-butanol was combined with other water-immiscible solvents. Table II summarizes the results of such combinations at a mixing ratio of 1:1. The addition of chloroform to 1-butanol leads to a 60% increase in PS-5 extractability.

For further improvement of the solvent extraction of PS-5, the mixing ratio of the 1-butanol–chloroform solvent was examined in detail. The per cent extractions of PS-5 in Fig. 2 are related with the 1-butanol concentration in the mixture. It is apparent that the extraction yield of PS-5 is practically the same in the concentration range of chloroform from 20 to 60%. Compound PS-5 is substantially insoluble in chloroform.

TABLE II

EFFECTS OF VARIOUS WATER-IMMISCIBLE SOLVENTS MIXED WITH 1-BUTANOL

Water-immiscible solvents were added to 1-butanol at a mixing ratio of 1:1. For details see Table I.

Solvent	*Per cent extraction*
Chloroform	82.1
Ethyl acetate	72.3
Benzene	62.5
Toluene	56.4
Methyl ethyl ketone	42.2
Diethyl ether	41.6
None	53.2

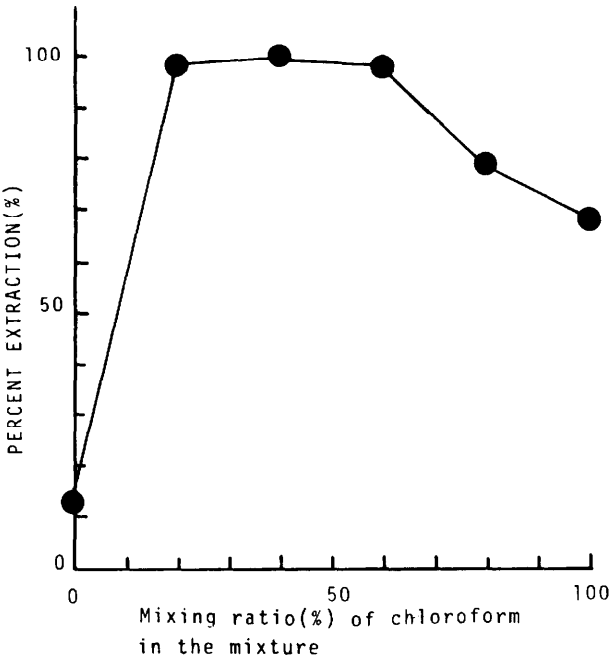


Fig. 2. Relation of the mixing ratio of 1-butanol and chloroform with the per cent extraction of PS-5. For details see Table I.

As carbapenem compounds rapidly decay under acidic conditions, it is very important to choose a practically usable pH value which is a compromise between the transfer efficiency and the chemical instability of carbapenem. The results in Fig. 3 indicate that compound PS-5 is satisfactorily extracted in the range pH 2–4.

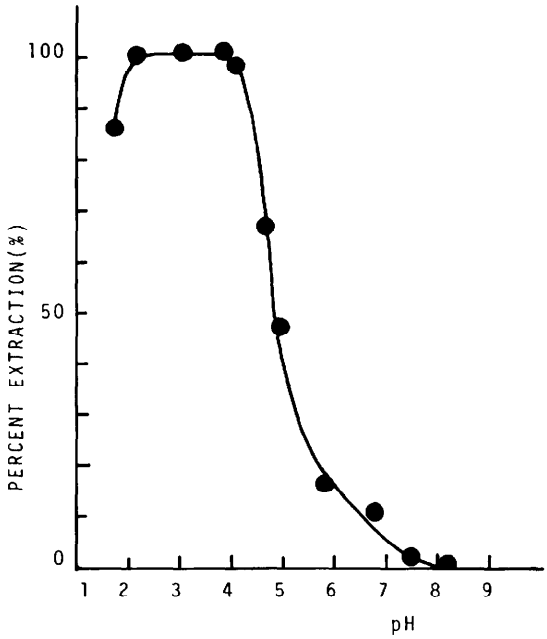


Fig. 3. Effect of pH on the extraction of PS-5 with 1-butanol–chloform (1:1). For details see Table I.

Among the known naturally occurring carbapenem compounds, PS-5, PS-6 and PS-7 are the easiest to extract with organic solvents, because they are the least hydrophilic. Epithienamycins A, B, C and D which were always produced in detectable amounts together with the PS series of carbapenems under our fermentation conditions were less extractable because of the 8-hydroxyl group.

Silica gel TLC system for carbapenem compounds

With quantitative analysis in mind, several mobile phases for silica gel TLC of carbapenem compounds were examined. For clear separation of PS-5 from other carbapenem compounds, acetonitrile–0.75% acetic acid (9:2) was found to be most suitable.

Authentic samples of PS-5, PS-6, PS-7 and epithienamycins A, B, C and D dissolved in 0.01 *M* phosphate buffer, pH 8.0, were spotted on a silica gel TLC plate and developed in the above mixture of acetonitrile and acetic acid. After visualization with the Ehrlich reagent, the carbapenem compounds gave the R_F values as presented in Fig. 4. We have not yet succeeded in separating the *cis/trans* isomers (that is, epithienamycins A and B from C and D respectively) by TLC.

Fig. 5 is a typical silica gel thin-layer chromatogram of the fermentation broth of *Streptomyces fulvoviridis* A933.

Quantitative measurement of carbapenem compounds on a silica gel TLC plate

As the carbapenem compounds listed in Fig. 1 have characteristic UV absorp-

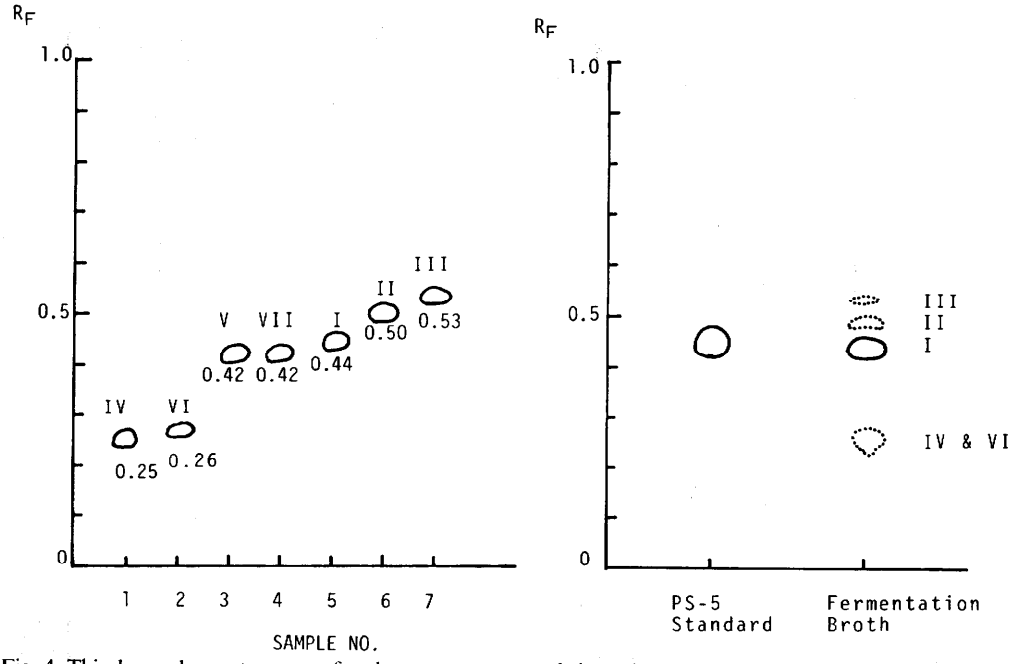


Fig. 4. Thin-layer chromatograms of carbapenem compounds investigated. Each carbapenem compound shown in Fig. 1 was extracted with 1-butanol–chloroform (1:1) at pH 3.0 and applied to a silica gel $60F_{254}$ plate. Mobile phase: acetonitrile–0.75% acetic acid (9:2). Detection: Ehrlich reagent, followed by heating at 100°C for 5 min. For chemical structures of components see Fig. 1.

Fig. 5. Thin-layer chromatograms of the fermentation broth of *Streptomyces fulvoviridis* A933. For chemical structures of components see Fig. 1.

tion peaks (301 nm for PS-5, PS-6 and epithienamycins A and C; 308 nm for PS-7 and epithienamycins B and D), authentic samples are clearly located under a UV lamp and can be quantitatied by UV spectroscopic densitometry. In practical analysis of fermenatation broths of carbapenem-producing streptomycetes, however, concomitant UV-absorbing impurities prevented spectrophotometric determination of carbapenem antibiotics on a silica gel TLC plate even after solvent extraction[7].

Naturally occurring carbapenem antibiotics are reported to react specifically with the Ehrlich reagent. Known quantities of carbapenem PS-5 on a silica gel plate were treated with the Ehrlich reagent at 100°C for 5 min and the colour intensities of the corresponding spots were read at 555 nm in a scanning densitometer. Fig. 6 shows the linear relationship of the amount of PS-5 with the densitometric peak area. Under the specified analytical conditions, we observed an acceptable linear relationship up to 10 μg per spot of PS-5.

To confirm the practical utility of this quantitative TLC analysis, we added known amounts of PS-5 to the fermentation broth of *Streptomyces fulvoviridis* A933 and examined the recoveries of PS-5. Fig. 7 summarizes the results, indicating that the present method is highly reliable, at least as far as compound PS-5 (and PS-6 and PS-7) is concerned.

Since the amounts of compounds V and VII in Fig. 1 are at most less than 1% of that of PS-5 in the fermentation broth of *Streptomyces fulvoviridis* A933[4] as pre-

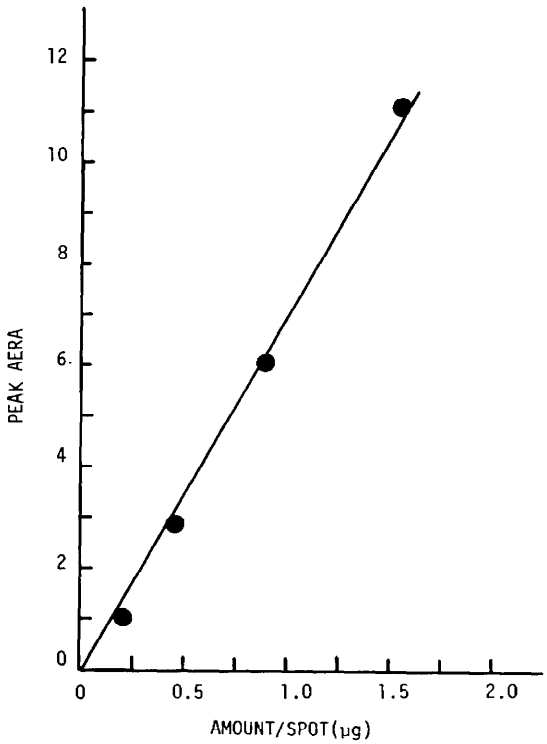


Fig. 6. Calibration line for the quantitation of PS-5 by TLC. The best-fitting straight line is calculated by means of the least squares method: peak area = $-0.05 + 7.10 \times$ (amount per spot) correlation coefficient = 0.9965.

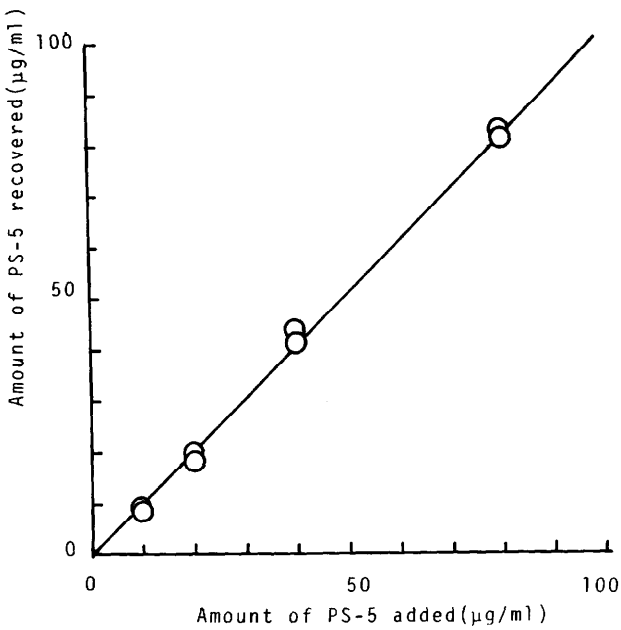


Fig. 7. Recovery of PS-5 from the fermentation broth of *Streptomyces fulvoviridis* A933. Authentic PS-5 was added to the fermentation broth after 24 h and then evaluated with the TLC method after extraction with *n*-butanol–chloroform (1:1).

viously noted, those compounds interfere little with the determination of PS-5 in the fermentation broth, even when they are nearly equal in R_F values as shown in Fig. 4.

As reported elsewhere[8], the routine use of this TLC analysis system has resulted in the discovery of a novel group of carbapenem compounds designated OA-6129A, OA-6129B_1, OA-6129B_2 and OA-6129C.

REFERENCES

1 E. Stahl (Editor), *Thin-layer Chromatography*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1969, p. 566.
2 K. Okamura, S. Hirata, Y. Okumura, Y. Fukagawa, Y. Shimauchi, K. Kouno, T. Ishikura and L. Lein, *J. Antibiot.*, 31 (1978) 480.
3 K. Okamura, A. Koki, M. Sakamoto, K. Kubo, Y. Mutoh, Y. Fukagawa, K. Kouno, Y. Shimauci, T. Ishikura and J. Lein, *J. Ferment. Technol.*, 57 (1979) 265.
4 N. Shibamoto, A. Koki, M. Nishino, K. Nakamura, K. Kiyoshima, K. Okamura, M. Okabe, R. Okamoto, Y. Fukagawa, Y. Shimauchi, T. Ishikura and J. Lein, *J. Antibiot.*, 33 (1980) 1128.
5 E. O. Stapley, P. J. Cassidy, J. Tunac, R. L. Monaghan, M. Jackson, S. Hernandez, S. B. Zimmerman, J. M. Mata, S. A. Currie, D. Doaust and D. Hendlin, *J. Antibiot.*, 34 (1981) 636.
6 K. Kiyoshima, unpublished results.
7 L. R. Treiber, *J. Chromatogr.*, 213 (1981) 129.
8 M. Okabe, S. Azuma, I. Kojima, K. Kouno, R. Okamoto, Y. Fukagawa and T. Ishikura, *J. Antibiot.*, 35 (1982) 1255.