Chem. Pharm. Bull.
28(3) 789—794 (1980)

Analytical Studies and Preparative Separation of Ansamitocins and Related Antibiotics Using High-Performance Liquid Chromatography

Motowo Izawa, Konomi Haibara, and Mitsuko Asai

Microbiological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd.[1]

(Received August 13, 1979)

Ansamitocins and related compounds were isolated from the culture broth of *Nocardia* sp. 1) The total amount of the antibiotics, after extraction with ethyl acetate from the culture broth, was determined by high-performance liquid chromatography on a μ Porasil column with a solvent mixture of methylene chloride–methanol (20: 1). 2) Each component in the mixture was quantitatively analyzed on a μ Bondapak column with 69% aqueous methanol. 3) Preparative separation of up to 5 g per cartridge column was carried out in good yield (90%) using a PrepLC/system 500 (Waters) and a column packing of Kieselgel 60 (E. Merck). The purities of the ASM obtained were above 95%.

Keywords——ansamitocin; antitumor; antibiotic; liquid chromatography; analytical separation; preparative separation

Ansamitocins and related compounds (antitumor antibiotics) were extracted with ethyl acetate from the culture broth of *Nocardia* sp. No. C-15003.[2] The extract, after purification by chromatography, gave five crystalline products, P–4, P–3′, P–3, P–2 (maytansinol propionate)[3] and P–1 (maytanacine),[3] of which P–4, P–3 and P–2 were the most abundant in ordinary fermentation broth. (It is important to determine the total amount of antibiotics in fermentation broth and the constituents of antibiotics obtained under particular culture conditions.)

These antibiotics have the same tricyclic skeleton consisting of a tetrasubstituted benzene, a cyclic carbinolamide and a macrocyclic aliphatic bridge with an ester side chain, as shown in Fig. 1. The only structural differences in these compounds are in the fatty acid moiety on the maytanside[4] C–3 ester. Clearly, separation is not likely to be easy in view of these

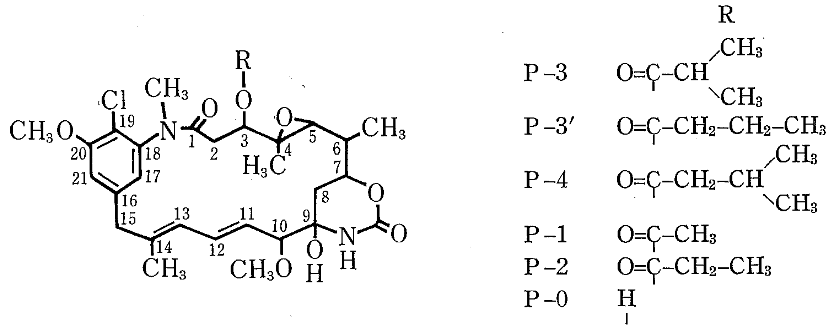

Fig. 1. Structural Formulae of Ansamitocins and Related Antibiotics

---

[1] Location: *17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532, Japan.*

[2] E. Higashide, M. Asai, K. Ootsu, S. Tanida, Y. Kozai, T. Hasegawa, T. Kishi, Y. Sugino, and M. Yoneda, *Nature* (London), **270**, 721 (1977).

[3] S.M. Kupchan, A.R. Branfman, A.T. Sneden, A.K. Verma, R.G. Dailey, Jr., Y. Komoda, and Y. Nagao, *J. Am. Chem. Soc.*, **97**, 5294 (1975); S.M. Kupchan, Y. Komoda, A.R. Branfman, A.T. Sneden, W.A. Court, G.J. Thomas, H.P.J. Hints, R.M. Smith, A. Karim, G.A. Howie, A.K. Verma, Y. Nagao, R.G. Dailey, Jr., V.A. Zimmerly, and W.C. Sumner, Jr., *J. Org. Chem.*, **42**, 2349 (1977).

[4] S.M. Kupchan, Y. Komoda, A.R. Branfman, R.G. Dailey, Jr. and V.A. Zimmerly, *J. Am. Chem. Soc.*, **96**, 3706 (1974).

relatively minor structural differences in large molecules. As we have reported already, ansamitocins (ASM) were separated by thin layer and conventional column chromatographic techniques from the mixed crystals obtained from the culture broth.[5] We next attempted to analyze each component of the mixed crystals and to separate ASM by a more convenient preparative liquid chromatography procedure.

In this paper, we describe two analytical separation methods utilizing high-performance liquid chromatography and preparative separation of ASM with a PrepLC/system 500 unit.

Experimental

Apparatus——Analytical liquid chromatography (ALC) was carried out using an ALC/GPC-244 machine (Waters Assoc., Milford, U.S.A.) equipped with a Corasil I precolumn (5 cm × 4 mm ID., Waters) and a μ Porasil column (30 cm × 4 mm ID., Waters) to determine the total amount of the antibiotics mixture and with a reverse-phase μ Bondpak C_{18} column (30 cm × 4 mm ID., Waters) to analyze each component, monitoring the UV absorption at 254 nm. Preparative liquid chromatography (PrepLC) was performed using a PrepLC/system 500 (Waters) equipped with a column packed with *ca.* 470 g of Kieselgel 60 (0.04—0.063 mm, E. Merck, Darmstadt, West Germany), which we prepared by placing the gel into an empty PrepPAK-500 cartridge case (30 cm × 5.7 cm ID., Waters) using the dry packing technique.

Elution Solvent——All solvents were of special grade (Wako Pure Chem., Osaka, Japan) and were used without further purification. For ALC, the eluant was a mixture of methylene chloride–methanol (20: 1) to measure the total amount of antibiotics and 69% aqueous methanol solution to analyze each component. A mixture of *n*-hexane–ethyl acetate (1: 7) saturated with water at 22° was used for PrepLC.

Material——The antibiotics were extracted with ethyl acetate from the culture broth, the extract was concentrated *in vacuo*, and the residue, after dilution with methylene chloride, was subjected to ALC to determine the total amount of the antibiotics. Mixed crystals and authentic samples of these antibiotics were obtained by the methods described in the previous paper.[5]

Results and Discussion

i) Measurement of the Total Amount of Antibiotics in the Culture Broth

The μ Porasil column was used for ALC of the antibiotics mixture. The eluant was a mixture of methylene chloride–methanol (20: 1). Fig. 2 shows a typical chromatogram of the separation of the antibiotics. The highest peak indicates the total amount of antibiotics. Several other peaks, which did not show any antifungal, antiprotozoal, or antitumor activity, were observed. Authentic samples of ASM P–4, P–3, and P–2 had the same retention times even when the sample amount was varied between 5 and 400 ng. Furthermore, the relative responses of these antibiotics to the UV detector were practically the same, because they have the same chromophore. The calibration curve for P–4, P–3 and P–2 is shown in Fig. 3. The coefficients of variation calculated from the peak height for injection and for the preparation of the sample solution by extraction from the culture broth are shown in Tables I and II, respectively. Table III shows the recovery yields of the additional antibiotics from the culture broth. The total amount of antibiotics could be determined satisfactorily by this method (Table IV).

ii) Quantitative Analysis of the Components of Mixed Crystals

The chromatographic conditions were studied using the μ Bondapak C_{18} column and 69% aqueous methanol solution as the eluant. The sample to be injected was dissolved in methanol. Fig. 4 shows a typical chromatogram of the separation of the components using a test mixture of authentic samples (P–2, 15%; P–3, 55%; P–4, 30%) in a ratio similar to that in the crystals obtained from a culture broth. The retention times of P–2, P–3 and P–4 were 4.25, 5.19 and 6.95 minutes, respectively, and were constant even when the sample amount was varied between 25 and 4000 ng under the conditions used. As the relative responses of these components to the UV detector (254 nm) were practically the same, their

---

[5] M. Asai, E. Mizuta, M. Izawa, K. Haibara, and T. Kishi, *Tetrahedron*, 35, 1079 (1979).

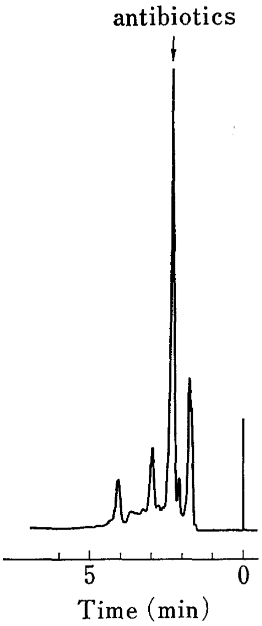

Fig. 2. Typical Chromatogram of the Extracted Antibiotics

Conditions: precolumn, Corasil I (0.037—0.05 mm, 5 cm × 4 mm ID.); column, μ Porasil (30 cm × 4 mm ID.); solvent, methylene chloride-methanol (20:1); flow rate, 2 ml/min; detector (254 nm) range, 0.1; sample size, 270 ng (antibiotics).

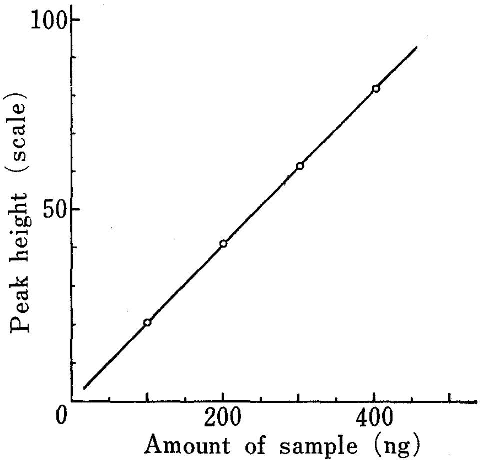

Fig. 3. Calibration Curve for P-4, P-3 and P-2

Conditions were the same as in Fig. 2.

Table I. Analytical Results for Standard Solutions[a]

Concentration μg/ml	Peak height (scale)									
	Found							Mean	[b]	[c]
20	20.0	19.7	20.0	20.1	20.0	19.6	19.6	19.9	0.21	1.08
40	40.6	38.5	40.0	39.8	38.8	38.7	39.2	39.4	0.78	1.98
60	60.2	59.6	59.6	59.7	60.3	59.8	58.9	59.7	0.46	0.77
80	79.4	78.2	79.2	77.7	80.5	79.5	79.1	79.1	0.91	1.15

[a] Mixture (P-2; 15%, P; 3; 55%, P-4; 30%) in CH_2Cl_2; injection, 5 μl.
[b] Standard deviation.
[c] Coefficient of variation.

Table II. Analytical Results for Sample Solutions obtained from the Culture Broth

Lot No.	Peak height (scale)				Standard deviation	Coefficient of variation
	Sample No.			Mean		
	1	2	3			
A	20.0	19.7	19.4			
	19.7	19.6	19.8			
	19.8	20.3	19.5			
	20.3	20.2	19.1			
	20.1	20.2	19.2			
Mean	20.0	20.0	19.4	19.8	0.35	1.75
B	29.3	30.3	29.4			
	29.6	29.8	30.2			
	29.7	29.6	29.8			
	28.6	29.9	30.3			
	28.8	29.4	29.8			
Mean	29.2	29.8	29.9	29.6	0.38	1.28

Table III. Recovery Yield of Additional Sample[a] from the Culture Broth

Lot No.	Amount of antibiotics in the culture broth μg/ml (X)	Amount of standard sample added to culture broth μg/ml (Y)	Amount of antibiotics μg/ml ($X+Y$)		Recovery yield %
			Calcd	Found	
A	19.8	35.7	55.5	54.5	98.2
B	29.6	35.7	65.3	62.9	96.3

a) Test mixture (P-2; 15%, P-3; 55%, P-4; 30%).

Table IV. Total Amount of Antibiotics in the Culture Broth

Lot No.	A	B	C	D	E
Total amount of antibiotics μg/ml	19.8±0.3	29.6±0.4	38.7±0.6	39.2±0.4	10.4±0.2

$n=3$.

relative ratios were calculated by the height-width method from the individual peaks. The test mixture was analyzed five times and the results obtained are listed in Table V. The relative ratio of mixed crystals obtained from the culture broth was determined by this method (Table VI, A-E).

iii) Preparative Separation of the Components

The eluant was a mixture of *n*-hexane–ethyl acetate (1: 7) saturated with water. To attain equilibrium in this chromatographic system, the eluant was passed through two columns packed with Kieselgel 60 (E. Merck) in series and the initial 1000 ml of effluent was purged, then the flow was stopped, and the effluent line was transferred to the eluant reservoir. The

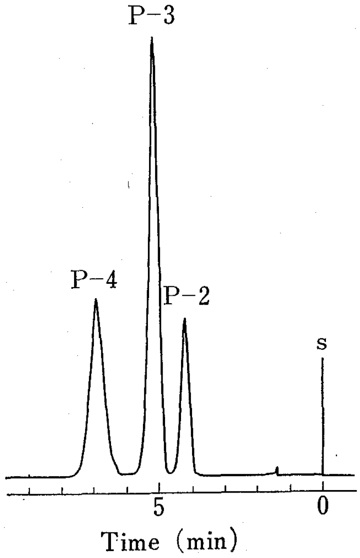

Fig. 4. Typical Chromatogram of a Test Mixture of the Antibiotics P-4, P-3 and P-2

Conditions: column, μ Bondapak C_{18} (30 cm × 4 mm ID.); solvent, 69% aqueous methanol; flow rate, 2 ml/min; detector (254 nm) range, 0.1; sample size 2 μg [test mixture of P-4 (30%), P-3 (55%) and P-2 (15%)].

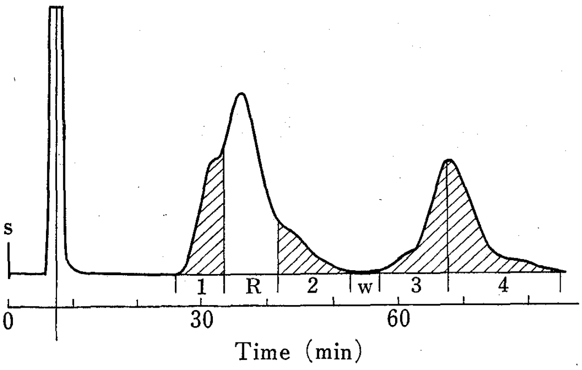

Fig. 5. Typical Preparative Chromatogram of a Mixture of P-4, P-3 and P-2

Conditions: column, one cartridge; solvent, hexane–ethyl acetate (1: 7) saturated with water; flow rate, 100 ml/min; detector response, 20; sample size, 1000 mg [test mixture of P-4 (30%), P-3 (55%) and P-2 (15%)]. Slanting lines show the collected fractions. Abbreviations R and W mean recycle and waste, respectively. Odd-numbered fractions contained P-4 and P-3 and even ones P-3 and P-2.

Table V. Analytical Results for a Test Mixture

Component of ASM	P-2	P-3	P-4 %
Ratio of the test mixture %	15	55	30
Found (%) repetition 1	14.7	54.7	30.6
Found (%) repetition 2	14.3	55.5	30.2
Found (%) repetition 3	15.2	55.1	29.7
Found (%) repetition 4	14.7	56.4	28.9
Found (%) repetition 5	14.6	56.0	29.4
Mean	14.7	55.5	29.8
Standard deviation	0.32	0.68	0.67
Coefficient of variation	2.20	1.23	2.24

Table VI. Constituents of Mixed Crystals and Crystals Obtained by PrepLC

Lot No.	P-2	P-3	P-4
A	15.3±0.2	55.8±0.3	28.9±0.3
B	16.7±0.2	61.2±0.3	22.1±0.4
C	15.4±0.3	62.4±0.5	22.2±0.3
D	15.8±0.2	63.8±0.4	20.4±0.3
E	16.6±0.3	62.4±0.5	21.0±0.3
F	99.2±0.2	0.8	0
G	2.4	96.9±0.3	0.7
H	0.3	2.0	97.3±0.3

$n=3$.

flow was allowed to resume, using 32 l of the eluant, and the eluant was allowed to recirculate until the baseline on the refractometer ceased to drift. The preparative separation was performed by a two-step method, namely, a sample containing P–4, P–3 and P–2 was separated into two fractions containing two components, (P–4 and P–3) and (P–3 and P–2), which were then separated into individual components. The sample to be injected was dissolved in 5 ml of methylene chloride per gram. A typical preparative separation chromatogram of a mixture of P–4, P–3 and P–2 is shown in Fig. 5. Separation could be scaled up to 5 g per cartridge column in this case. Figs. 6 and 7 are typical chromatograms of the preparative separation of mixtures of P–4 and P–3, and P–3 and P–2, respectively, obtained by the method described in the legend to Fig. 5. Fractions from the final run were investigated by TLC. As a further purity check, we combined appropriate fractions and checked them by ALC. The purities of the components thus obtained were above 95% (Table VI, E-H). The recovery yield of each component after two steps was about 90%.

First, we tried the following procedure to find a suitable chromatographic system for PrepLC. The antibiotics mixture was separated on a Waters analytical liquid chromatograph with a μ Porasil column using a refractometer detector. When the chromatographic conditions had been optimized, the separation was simply scaled up to the PrepLC level using a PrepPAK-500/silica cartridge (Waters). A mixed organic solvent containing water (a mixture of *n*-hexane, ethyl acetate and water) was used as the eluant. In this chromatographic system, semipreparative separation of a 5-mg sample load on a μ Porasil column was efficient, but scale-up to a 500 mg sample load could not be achieved with the PrepLC-500/silica cartridge. Therefore, we repacked an empty PrepPAK-500/silica cartridge with Kieselgel 60 (E. Merck) using a dry packing method. This packed cartridge was suitable for preliminary separation of a 500 mg sample load using the solvent used above.

Finally, an easily useable chromatographic eluant for the packed cartridge was sought. The solvent conditions were investigated by TLC (Kieselgel 60 F_{254}, 0.25 mm, E. Merck) and

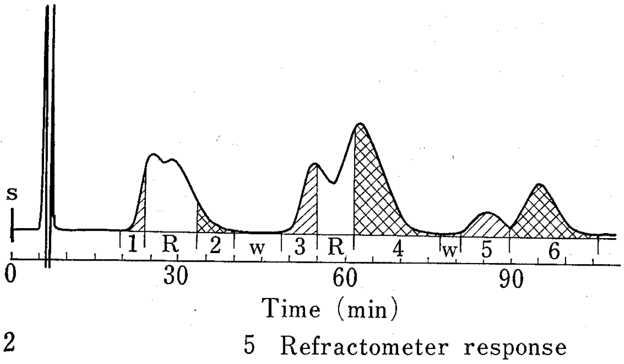

Fig. 6. Typical Preparative Chromatogram of P-4 and P-3

Conditions: column, two cartridges in series; flow rate, 150 ml/min; sample size, 900 mg [a mixture of P-4 (37.4%) and P-3 (62.6%) obtained by the method described in the legend to Fig. 5]. Other conditions and abbreviations were the same as in Fig. 5. Odd-numbered fractions contained P-4 and even ones P-3.

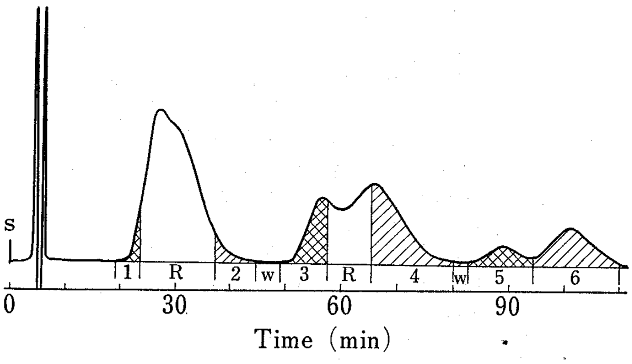

Fig. 7. Typical Preparative Chromatogram of P-3 and P-2

Conditions: detector response, 5; sample size, 5000 mg [a mixture of P-3 (38.3%) and P-2 (61.7%) obtained by the method described in the legend to Fig. 5]. Other conditions and abbreviations were the same as in Fig. 5. Odd-numbered fractions contained P-3 and even ones P-2.

scaled up directly to the PrepLC level using the same solvent. The *Rf* value on TLC for P-3 was adjusted to about 0.3 by changing the solvent ratio in view of the amount of solvent, time needed and sample load to be used for PrepLC. We finally selected a mixture of *n*-hexane–ethyl acetate (1: 7) saturated with water for PrepLC; the *Rf* values on TLC were 0.36 (P-4), 0.31 (P-3), and 0.27 (P-2) in this solvent.

As shown in Figs. 5, 6, and 7, preparative separations could be performed effectively in this chromatographic system. The differences of activity, particle size and surface area of silica gels employed were thought to be the main factors affecting the efficiency of separation in this case.

Acknowledgement We would like to express our thanks to Drs. E. Ohmura, M. Yoneda and T. Kishi for their encouragement and discussions throughout these studies. We also thank Mr. S. Tanida and the staff members in charge of fermentation and extraction for their cooperation.