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Maridomycin, a New Macrolide Antibiotic. XII.[1] A Simple Quantitative Analysis of 9-Propionylmaridomycin Components

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Macrolide antibiotic producing organisms produce usually several homologous components. A quantitative analysis of these components has not been reported because of their physicochemical similarity.

A method for quantitative analysis of a mixture of macrolide components was established by the use of thin-layer chromatography and ultraviolet (UV) absorption spectrometry.

9-Propionylmaridomycin which has no absorption maximum in UV and visible region was converted into its thiosemicarbazone exhibiting an absorption maximum at 271 nm.

The thiosemicarbazones formed are separated by silica gel thin-layer chromatography using a solvent system of hexane–isopropyl ether–EtOH–H_2O (1 : 4 : 3 : 2). The composition ratio of each component was calculated from the absorbance of each extract of separated fraction. By this method recovery rate was almost quantitative.

1. Introduction

Maridomycins (MDM) consisting of six components were obtained from the culture filtrate of *Streptomyces hygroscopicus* No. B-5050, and named as maridomycin I, II, III, IV, V, and VI, respectively. All of these components show similar physicochemical, chemical and biological properties.[3,4]

9-Propionylmaridomycins (PMDM), obtained[5] from MDM by selective propionylation of hydroxyl group at position 9, showed improved blood level, chemotherapeutic effect on mice and less acute toxicity on mice.[6,7] Macrolide producing organisms produce occasionally several homologous components. A quantitative analysis of these components was difficult because of their physicochemical similarity. For the separation and detection of these components, paper chromatography and silica gel thin-layer chromatography were applied.[4] This paper deals with a simple quantitative analysis of PMDM components using thiosemicarbazone formation and their silica gel thin-layer chromatography (TLC).

The structure of PMDM-18-thiosemicarbazone was determined from its elemental analysis, specific rotation, molecular formula, ultraviolet (UV)-, infrared (IR)-, and nuclear magnetic resonance (NMR)-spectrum as shown below.

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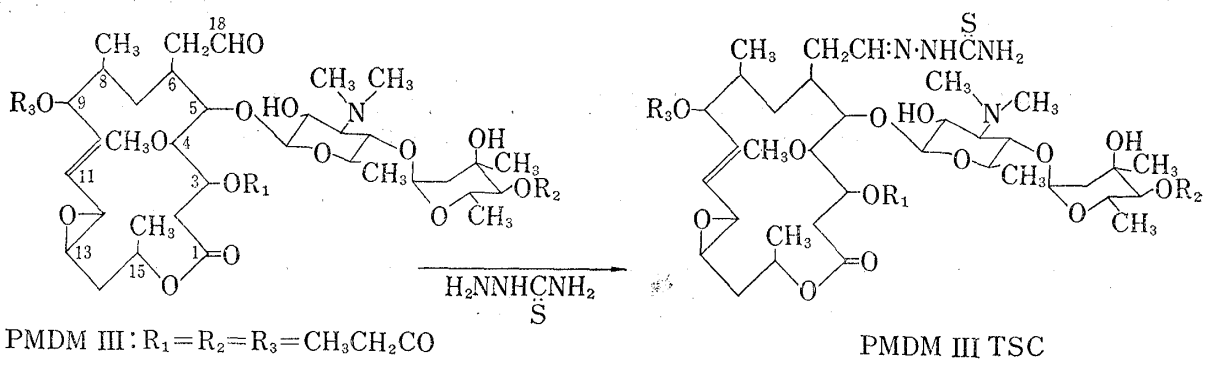

Chart 1

2. Materials and Methods

2-1. Reagents and Apparatus——PMDM and six components of PMDM were prepared in this laboratory. Thiosemicarbazide was purchased from Wako Pure Chemicals. Precoated plate, Spotfilm, Silica gel f, 20×20 cm (Tokyo-Kasei Co.) was used for thin-layer chromatography. Upper layer of a mixture of hexane–isopropyl ether–ethanol–H_2O (1:4:3:2) was used as the solvent system. A Beckmann Model DU spectrophotometer was used for the determination of absorbances.

2-2. Synthesis of 9-Propionylmaridomycin III Thiosemicarbazone (PMDM III TSC)——300 mg of PMDM III and 60 mg of thiosemicarbazide dissolved in 10 ml of EtOH were refluxed for 5 hr. After cooling, the reaction mixture was concentrated *in vacuo* to dryness. The residue was dissolved in $CHCl_3$, filtered and concentrated. The concentrate was chromatographed on 20 g of silica gel (Merck 0.2—0.05 mm) using a solvent system of $CHCl_3$–MeOH (80:1). The fractions of PMDM III TSC were collected, evaporated and the residue was recrystallized from EtOH, giving 188 mg of pure PMDM III TSC as colorless plate, mp 146—147°.

2-3. Standard Analytical Procedure——Weigh 50 mg of PMDM and 10 mg of thiosemicarbazide in 5 ml of EtOH and reflux for 1 hr. After cooling, pipette 150 μl of the reaction mixture and apply on a silica gel plate (20×20 cm) as 7 cm band. Take 50 mg of standard pure PMDM III and 1 mg of thiosemicarbazide independently, and treat similarly as above. Pipette 5 μl of the reaction mixture and apply on the same plate horizontally, 1.5 cm separated from the sample.

Develop the plate in a glass box with a solvent system of hexane–isopropyl ether–EtOH–H_2O. When the front of solvent developed 12 cm from the origin, take off the plate and air-dry at room temperature. Then submit to repeated TLC using the same solvent system and dry. Mark the separated zone under an ultraviolet lamp (253.6 nm). Calculate the *Rf* value of each component of PMDM TSC relative to standard PMDM III TSC and identify as each component of PMDM thiosemicarbazones (I′—VI′).

Table I. *Rs* Values of Each Component of PMDM TSC

Component of PMDM TSC	I′	II′	III′	IV′	V′	VI′
Rs value	1.4	1.2	1.0	0.8	0.7	0.6

$$Rs = \frac{Rf \text{ value of each separated zone of sample}}{Rf \text{ value of standard PMDM III TSC}}$$

Scrape off the silica gel from each zone separately, shake vigorously with EtOH for 30 min and separate by centrifugation. After taking supernatant, repeat shaking with EtOH and separation. Take each supernatant and dilute with EtOH to adjust concentration (5 μg/ml—25 μg/ml). The solutions are taken as sample solutions (T_I'—T_{VI}'). Independently, scrape off silica gel from the same *Rs* value zone, treat as above and make each supernatant as blank test solution (B_I'—B_{VI}'). Determine the absorbance at 271 nm of each sample (T_I'—T_{VI}') and blank solution (B_I'—B_{VI}'). Calculate the difference of absorbances between the sample and the blank solution, respectively (E_I'—E_{VI}'). Determine the composition ratio of each component as percentage. The total of E_I' to E_{VI}' is 100%.

3. Results and Discussions

3-1. Physicochemical Properties of PMDM III TSC

PMDM III TSC synthesized in 2—2 showed the following physicochemical properties. mp 146—147°, $[\alpha]_D^{27}$ −77.3° (c=1.02 in $CHCl_3$). *Anal.* Calcd. for $C_{45}H_{74}O_{16}N_4S\cdot 3H_2O$: C, 53.34; H, 7.96; N, 5.53; S, 3.16. Found: C, 53.59; H, 7.74; N, 5.47; S, 3.20. UV λ_{max}^{MeOH} nm (ε): 271.0 (24500). IR ν_{max}^{KBr} cm^{-1}: 1595 (δ_{NH_2}), 1520 (ν_{CS}). An aldehyde band of PMDM III at 2725 cm^{-1} disappeared in PMDM III TSC. NMR (100 MHz, in d_6-acetone) δ (ppm): 7.16 (1H, NH), 7.35 (1H, NH), 10.12 (1H, NH), 7.66 (1H, t, J=6 Hz, $-CH_2-CH=N-NH$). An aldehyde proton of PMDM III at 9.62 ppm (1H, s) disappeared in PMDM III TSC. All three protons (NH) disappeared after addition of D_2O.

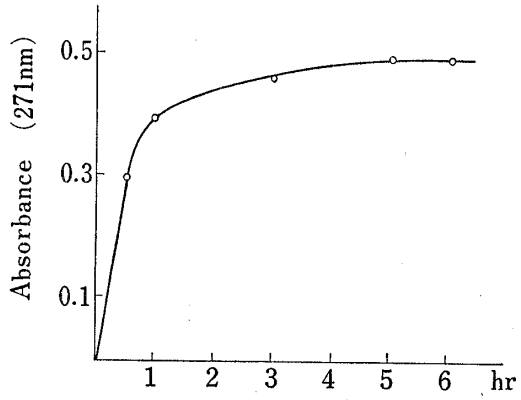

Fig. 1. Relationship between Reaction Time and PMDM III TSC Formation

3-2. The Structure of PMDM III TSC

From these data together with the fact that the structure of MDM II TSC has been elucidated by the authors,[8] the structure of PMDM III TSC was determined as shown in Chart 1.

3-3. Relationship between Concentration and Absorbance of PMDM III TSC

The pure crystal of PMDM III TSC was weighed accurately and made 0, 5, 10, 15, 20, 25, 30 μg/ml EtOH solution. From the absorbances determined at 271 nm it was confirmed that the solution of PMDM III TSC obeyed the Beer's law from 5 μg/ml to 30 μg/ml.

3-4. Reaction Time

The relationship between the reaction time and formation of PMDM III TSC was examined. As shown in Fig. 1 the reaction rate becomes approximately constant after 5 hr, but the reaction time was set for 1 hr to avoid the formation of by-products. It is enough for the analysis of composition ratio of each component if each component forms the thiosemicarbazone in the same reaction rate. To confirm the same reaction rate almost equal weight of each pure component of PMDM was mixed and analyzed according to the standard analytical procedure (2-3). As shown in Table II, satisfactory results were obtained. The reaction time was set for 1 hr in the procedure.

Table II. Analytical Result of the Mixture

Component of PMDM		I	II	III	IV	V	VI
Ratio of the mixture (%)		15.7	18.3	17.9	16.2	16.8	15.1
Found (%) repetition	1	16.4	17.9	18.7	15.5	16.2	15.3
	2	14.8	19.2	18.5	17.0	16.1	14.4
	3	16.1	17.6	17.0	15.9	17.6	15.8
mean		15.8	18.2	18.1	16.1	16.6	15.2
Δ (Found-Calcd.)		0.1	0.1	0.2	0.1	0.2	0.1
R/Mean (R=Max-Min)		0.10	0.09	0.095	0.095	0.09	0.093
Standard deviation (σ)		0.85	0.85	0.93	0.78	0.84	0.71

8) M. Muroi, M. Izawa, H. Ono, E. Higashide, and T. Kishi, *Experientia*, **28**, 878 (1972).

3-5. Thin-Layer Chromatography

(1) **TLC-Plate and Solvent System**——Spot film silica gel f (Tokyo-Kasei Co.), Woelm precoated silica gel $F_{254/366}$ (Woelm) and Kiesel Gel HF (Merck, 400 μ) gave good separation of six components of PMDM TSC using a solvent system of upper layer from hexane–isopropyl ether–EtOH–H_2O (1: 4: 3: 2) mixture. Thin-layer chromatogram of the reaction mixture of PMDM or thiosemicarbazide alone is shown in Fig. 2.

(2) **Developing Distance and Repetition of the Procedure**——For good separation of each component, repeated ascending method is recommended using the same solvent system as shown in Fig. 3. In general, better separation of the two components is obtained by the repeated TLC when the sum of the *Rf* values of the two components, Rf_1+Rf_2, is less than 1.0, which is mathematically elucidated.

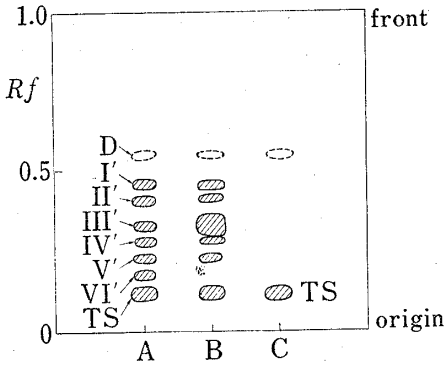

Fig. 2. Thin-layer Chromatogram of Reaction Mixture

A: reaction mixture of equal weight of each component
B: reaction mixture of a lot
C: reflux solution of thiosemicarbazide (TS)
D: degradation product of thiosemicarbazide

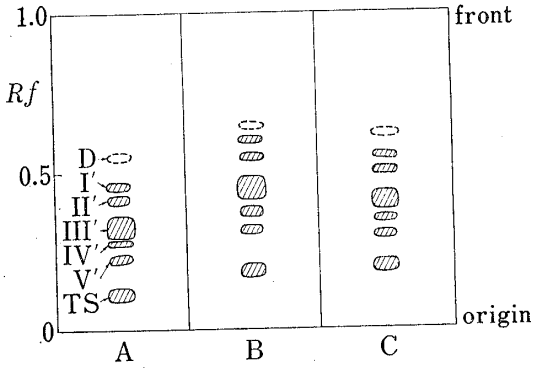

Fig. 3. Thin-layer Chromatogram of Reaction Mixture

A: developed 15 cm once
B: developed 15 cm twice
C: developed 12 cm twice
D: degradation product of TS
TS: thiosemicarbazide

(3) **Solvent and Shaking Time for Extraction from Silica Gel**——Effects of the volume of solvent and shaking time on the recovery rate of PMDM TSC from silica gel were investigated. As shown in Table III, the satisfactory result was obtained in the case of twice extraction (30 ml, 15 ml) and 30 min shaking, respectively.

Table III. Effects of Solvent and Shaking Time on the Recovery of PMDM TSC

Solvent	Volume (ml)	Shaking time (min)	Rate of extraction (%)
MeOH	5	30′	68.9
MeOH	30	30′	97.7
MeOH	30+15	30′+30′	99.9
EtOH	5	30′	84.8
EtOH	30	30′	98.3
EtOH	30+15	30′+30′	101.0

EtOH was chosen as the extraction solvent because of its more efficient extraction and higher boiling point than those of MeOH.

3-6. Calibration Curve for PMDM III TSC

1—40 mg of PMDM III was reacted with thiosemicarbazide. The absorbance of the extracted solution from silica gel was determined as described above (2-3). The absorbance was calculated as in 50 ml of EtOH solution. The relationship between the calculated absorbance and the concentration of PMDM III TSC showed the almost straight line through zero

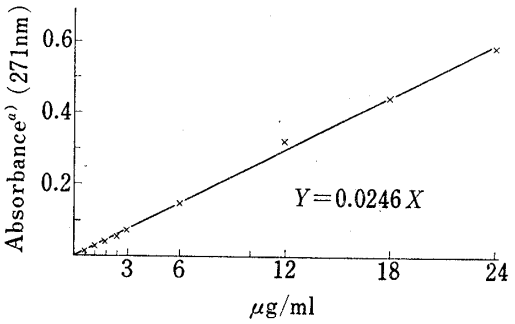

Fig. 4. Calibration Curve of PMDM III TSC

Y: absorbance
X: concentration of PMDM III TSC
a) Absorbances are calculated as in 50 ml from the observed absorbances which are determined in the concentration of 5—25 μg/ml solution.

point as shown in Fig. 4. This result was consistent with the data obtained with the standard sample of PMDM III TSC. The minimum concentration determined was 0.6 μg/ml.

3-7. Quantitative Analysis of the Mixture

The synthetic mixture of known amounts of PMDM I—V was analyzed by the standard procedure (2-3). The composition ratio was divided into three groups as component III, component V, and component I, II, and IV. It was found that the accurate ratio is obtained (Table IV) even if one component is predominant as compared with other components.

3-8. Composition Ratio of a Sample

By the standard procedure (2-3), satisfactory results were obtained for a sample of PMDM as shown in Table V.

TABLE IV. Composition Ratio of Each Group in the Case of the Mixture

Group		III	V	I+II+IV		
The Mixture	ratio of each component	III	V	I	II	IV
		81.4%	3.8%	3.2%	3.8%	7.8%
	ratio of each group	81.4	3.8		14.8	
Found	repetition 1	80.0	3.8		16.2	
	2	82.1	3.4		14.5	
	3	80.8	4.1		15.1	
	mean	81.0	3.8		15.2	
Δ (Found-Calcd.)		0.4	0		0.4	
R/Mean (R=Max-Min)		0.026	0		0.11	
Standard deviation (σ)		1.06	0.35		0.86	

TABLE V. Analytical Data of a Sample

Component group		III	V+VI	I+II+IV
Found	repetition 1	83.4%	4.2%	12.4%
	2	84.7	3.8	11.5
	3	82.6	4.5	12.9
	mean	83.6	4.2	12.3
Range (R)		2.1	0.7	1.4
(R/Mean)		0.025	0.17	0.11
Standard deviation (σ)		1.06	0.35	0.71

4. Conclusion

The structural differences of homologous components of macrolide antibiotic are derived from the difference of number of carbon chain in fatty acid ester moieties. Each component shows similar physicochemical properties and the quantitative analysis of them has not been reported. Eight components of leucomycins produced by *Streptomyces kitasatoensis* were

separated by high speed liquid chromatography, but the quantitative analysis of these components has not been described.[9]

In this report, PMDM which shows no characteristic ultraviolet absorption maximum was converted into its thiosemicarbazone exhibiting an absorption maximum at 271 nm. The reaction mixture was separated on a silica gel plate and each component was extracted. The composition ratio was calculated from the absorbance of each extracted solution. The analysis of the mixture of each component and repeated test of sample indicated that this procedure is an excellent simple quantitative analytical method.

It is suggested that this method will be applicable to all of sixteen membered basic macrolide antibiotics which have an aldehyde group in their aglycone moieties. For example, each component of natural maridomycins was separated using a solvent system of isopropyl ether–EtOH–H_2O (5 : 3 : 2).

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