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ANALYTICAL STUDIES OF MARIDOMYCIN

II. SEPARATION OF 9-PROPIONYLMARIDOMYCINS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Propionyl derivatives of maridomycins, 9-propionylmaridomycins (PMDMs), are sixteen-membered ring macrolide antibiotics of six analogous components: I, II, III, IV, V and VI. The present paper deals with the separation and quantitative analysis of these components. The analysis was performed by thin-layer chromatographic separation, addition reaction of gaseous iodine with PMDMs on the plate, extraction of the PMDM–iodine complexes, and subsequent analysis of the amount of reacted iodine, using an automatic analysis system. To ascertain the reaction product of this system, PMDM III–iodine complex was synthesized separately under a liquid-phase reaction and the ratio of PMDM III and iodine atoms was determined to be 1:3 on physicochemical examination.

INTRODUCTION

To improve their antibiotic activity, 9-propionylmaridomycins (PMDMs) were derived from maridomycins (MDMs). Since the MDMs consist substantially of six analogous components, PMDMs obtained by reaction of MDMs with propionyl-chloride[1] also have six analogous components: I, II, III, IV, V and VI. As the antimicrobial activities of these components are different from each other[1], separation is necessary for chemical and biological evaluation of PMDMs. I have separated these components by high-performance liquid chromatography[2], but a simpler and more efficient method for routine work was needed. In this paper, PMDMs were separated by thin-layer chromatography (TLC), quantitatively combined with iodine, then subjected to an automatic analysis system after the iodine had been extracted in an alkaline solution. The structures of PMDMs were shown in a previous paper[2].

EXPERIMENTAL

Materials

Authentic samples of PMDMs were synthesized[1] and separated by silica gel chromatography. All reagents used in this investigation were of special grade and purchased from Wako (Osaka, Japan).

Adsorbent layer

Silica gel G Type 60 (E. Merck, Darmstadt, G.F.R.) was coated, 0.25 mm thick, on a glass plate (20 × 20 cm) using a commercial spreader (Desaga, Heidelberg, G.F.R.). The plates were dried overnight at room temperature, then activated at 110° for 2 h.

Developing solvent

The upper layer of a mixed solvent of *n*-hexane–diisopropyl ether–ethanol–water (3:20:5:4) was used.

Instruments

Colorimetric determination of the iodine was performed with a Technicon AutoAnalyzer. Fig. 1 shows the flow diagram of the analysis system[3].

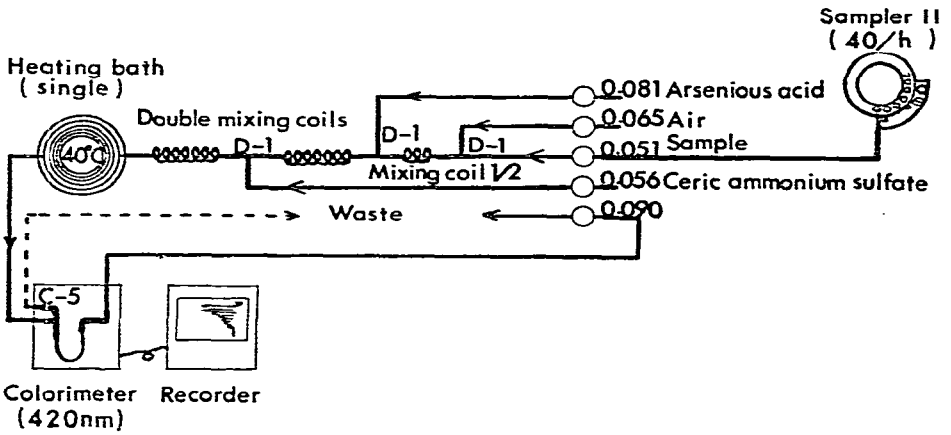


Fig. 1. Flow diagram of the automatic iodine analysis system.

Thin-layer chromatography

The samples containing about 25 mg of PMDMs were weighed and dissolved in 1 ml of chloroform. Using a microsyringe, 20 μl were applied to the adsorbent layer in the shape of a band (about 1 cm long), 2 cm above the bottom edge. The layer was developed in a normal chamber without pre-saturation with solvent vapor until the mobile phase had travelled 15 cm. After evaporation of the solvent, the plates were placed in an atmosphere of iodine vapor for 30 min. The visualized spots were delineated, then the plates were put in an air current (2–3 m/sec) for 1 h to remove excess iodine. Each spot was scraped off with a spatula and transferred into a test tube. An adjacent area of the plate was treated in exactly the same way to

obtain the blank value. Next, 5 ml of 0.1 *N* sodium hydroxide were added to the test tubes, which were shaken for 10 min then centrifuged at 1400 *g* for 5 min. The supernatants (1 ml) were put in volumetric flasks, which were then filled to the scale line with distilled water.

Determination of the iodine contents

About 2 ml of the above solutions were poured into sample cups of the AutoAnalyzer. The cups were placed in the holes of the Sampler plate alternately with those filled with distilled water. Standard samples of iodine solutions of 0–253.8 ng/ml (0–1 nmole/ml) were simultaneously determined with the above samples for each investigation. The principle of this determination is the iodine catalysis ceric–arsenite reaction modified from the Technicon AutoAnalyzer PBI procedure[3]. The contents of each component were calculated from the iodine contents where one mole of PMDM corresponds to 1.42 moles of iodine.

RESULTS

PMDM is generally comprised of over 75% of III, less than 20% of I + II + IV, and less than 5% of V + VI. A typical thin-layer chromatogram of crude PMDM, which was prepared from a fermentation broth of *Streptomyces hygroscopicus* No. B-5050 according to Ono *et al.*[4], was compared with that of a mixture of the authentic samples (Fig. 2). As the separation of these components deteriorated if the layers were presaturated with the vapor of the developing solvent, there was no pre-saturation of the developing chamber.

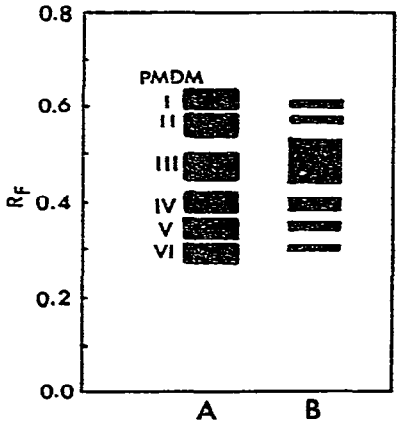


Fig. 2. Typical thin-layer chromatograms of PMDMs. A = a mixture of equal weights of six components of PMDM. B = crude PMDM. Conditions: plate, Merck silica gel G type 60; solvent, upper layer of a mixture of *n*-hexane–diisopropyl ether–ethanol–water (3:20:5:4); sample size, 500 μg; detection, iodine vapor.

To determine the reaction conditions of PMDMs and iodine, PMDM III (main component of PMDM) was used for subsequent experiments.

The time of exposure to the iodine vapor was determined after removing the excess iodine and calculating the weight of reacted iodine. As the amount of iodine

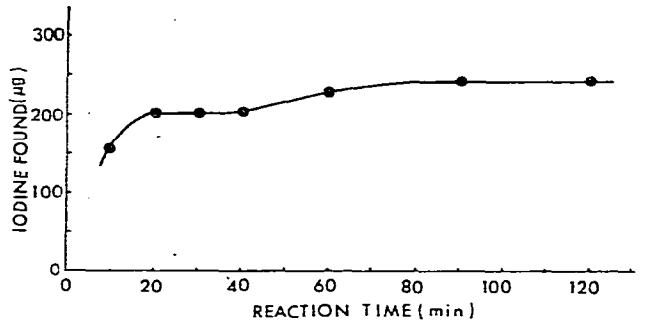


Fig. 3. Effect of time on the amount of iodine reacted in the iodine-saturated chamber. Sample: PMDM III (500 μg). Excess iodine was vaporized in an air current at 25° for 1 h. For details, see text.

reacted was constant between 20 and 30 min, then gradually increased with time (Fig. 3), the time of exposure to iodine was limited to 30 min.

Sufficient excess iodine was removed after 1 h (Fig. 4), and the color of the spots changed from brown to pale yellow.

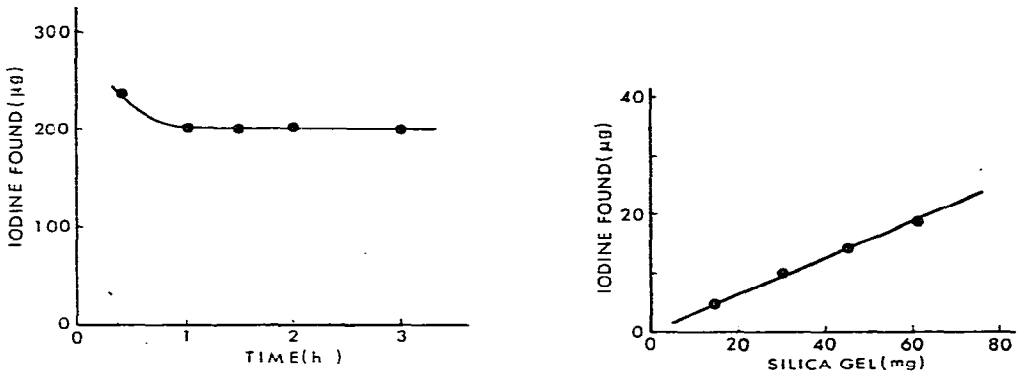


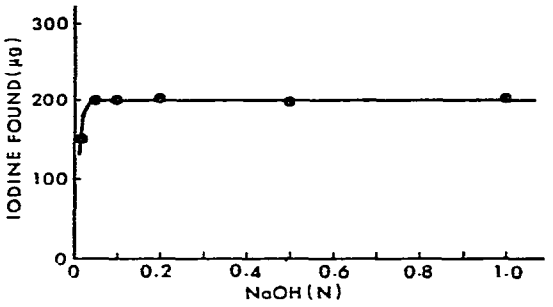
Fig. 4. Effect of time on the removal of excess iodine. Sample: PMDM III (500 μg). Reaction time in the iodine-saturated chamber was 30 min. For details, see text.

Fig. 5. Comparison of the weight of the silica gel scraped from the plate with the amount of iodine found.

The blank values of the colorimetric determinations were proportional to the amounts of silica gel scraped from the plate (Fig. 5). Thus, the same amount of blank layer had to be scraped from the plate and treated in the same way as the sample.

The concentration of the sodium hydroxide solution used for extraction did not affect the result unless it was less than 0.05 *N* (Fig. 6).

The iodine contents of the extracts were plotted against the amounts of PMDM III or IV. The calibration curve obtained was linear up to 800 μg of PMDMs as shown in Fig. 7. In this situation, 443 μg (0.5 μmoles) of PMDM III gave 180 μg (0.71 μmoles) of iodine in contrast with the calculated value of 443 μg of PMDM III



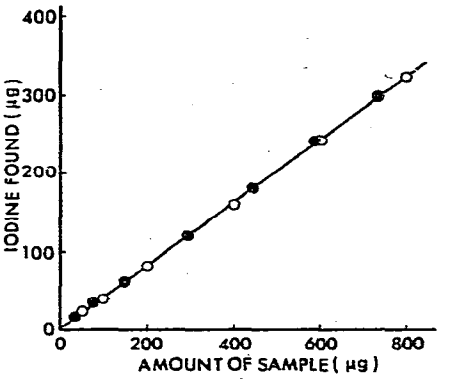


Fig. 6. Effect of the sodium hydroxide concentration used in extracting PMDM III–iodine complex from the TLC plate.

Fig. 7. Linear relationship between sample amount and iodine found. ●, PMDM III; ○, PMDM IV.

corresponding to 190 μg iodine (0.75 μmoles). The accuracy of the result was calculated from data determined 12 times using six plates with the same batch of PMDM III, which is the main component of the PMDM mixture; the coefficient of variation was $\pm$1.9% (Table I). The artificial mixture of known amounts of PMDM III, IV and V was analyzed by the present method, and satisfactory results were obtained as shown in Table II.

TABLE I

RESULTS FROM DIFFERENT PLATES OF THIN-LAYER CHROMATOGRAPHY
Sample size: PMDM III, 500 μg.

Plate No.	*Iodine found (μg)* *
1	202.5
2	197.5
3	195
4	200
5	205
6	203
Mean	200.5
Standard deviation	3.7
Coefficient of variation (%)	1.9

* Mean of two determinations.

TABLE II

ANALYTICAL DATA OF A MODEL SAMPLE OF KNOWN COMPOSITION

	Composition of PMDM (%)		
	III	*IV*	*V*
Artificial mixture of III, IV, V	80.9	14.0	5.1
Test 1	81.9	14.0	4.1
2	80.5	15.1	4.4
3	79.6	14.4	6.0
4	78.7	15.9	5.4
Mean	80.2	14.8	5.0

DISCUSSION

Šaršúnová *et al.*[5] reported that tertiary-bonded nitrogen forms a complex by addition of three iodine atoms to the following structure:

$$\equiv N^{+}\text{–}H + I_3^-$$

I assumed that the iodine complex in this report had the same structure as above, because the molar ratio of PMDM III to iodine (1:1.42) was similar to that of Šaršúnová *et al.* (1:1.5). The fact that the molar ratio was not closer to that of Šaršúnová *et al.* may have been due to the presence of water of crystallization[6] (PMDMs generally contain two H_2O groups in a molecule). However, the good precision in repeated experiments indicated the acceptability for quantitative analysis.

To determine the bonding ratio of PMDMs and iodine in the above complex, one mole ($1.13 \cdot 10^{-3}$ *M*) of PMDM III was reacted with 1.5 moles of iodine (1.7×10^{-3} *M*) in 70 ml of 90% methanol at room temperature for 20 h. After the evaporation of the solvent, the residue was dissolved in 67 ml of mixed solvent, which was the upper layer of *n*-hexane–diisopropyl ether–ethanol–water (5:20:9:10) to which 10% of ethanol had been added, then the solution was left standing for a week at room temperature. Brown needle crystals were obtained (about 48% yield). The physicochemical characteristics of these crystals were; m.p.; 147–151°: elemental analysis (Calc. for $C_{44}H_{72}NO_{17}I_3$ C, 41.69; H, 5.72; N, 1.10; I, 30.03%). Found: C, 41.75; H, 5.59; N, 1.03; I, 29.24%. UV $\lambda_{max}^{CHCl_2CHCl_2}$: 292 nm ($\varepsilon = 38700$); nuclear magnetic resonance: the signal of methyl in the dimethylamino group shifted from 2.52 ppm to 3.27 ppm (in $CDCl_3$). Treatment of these crystals with 0.01 *N* sodium hydroxide produced PMDM III. These results indicated that the complex of PMDM III and iodine should have PMDM III triiodide. The same result was obtained by X-ray analysis[7].

The present method could be applicable to other macrolide antibiotics such as leucomycins, carbomycins, and erythromycins.

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REFERENCES

1 S. Harada, M. Muroi, M. Kondo, T. Tsuchiya, T. Matsuzawa, T. Fugono, T. Kishi and J. Ueyanagi, *Antimicrob. Ag. Chemother.*, 4 (1973) 140.
2 K. Kondo, *J. Chromatogr.*, 169 (1979) 329.
3 *Technicon AutoAnalyzer Methodology Bulletin* N-56 (1969).
4 H. Ono, T. Hasegawa, E. Higashide and M. Shibata, *J. Antibiot.*, 26 (1973) 191.
5 M. Šaršúnová, B. Kakáč and L. Krasnec, *J. Chromatogr.*, 48 (1970) 353.
6 Y. Akagi, S. Takamoto and T. Matsuzawa, *J. Takeda Res. Lab.*, 32 (1973) 62.
7 K. Kamiya, Y. Wada and K. Kondo, *Chem. Pharm. Bull. (Tokyo)*, 26 (1978) 1753.