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Note

Analytical studies of maridomycin

III. Spectrophotometric determination of maridomycins and 9-propionylmaridomycins as iodine complexes after thin-layer chromatography

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Maridomycins (MDMs), isolated from the culture filtrate of *Streptomyces hygroscopicus* No. B-5050, consist of six components, namely MDM I, II, III, IV, V and VI[1]. 9-Propionyl derivatives of MDMs (PMDMs), which were prepared to improve their bioavailabilities, also have six analogous components. During the determination of the ratios of each component, it was found that PMDM reacted with iodine to form a complex, $[PMDM–H]^+I_3^-$. Using this characteristic reaction, an analytical method was established, *i.e.*, PMDMs were separated by thin-layer chromatography (TLC) and treated with gaseous iodine on the plate. After removal of the excess iodine, the reacted iodine extracted from the plate was measured by the ceric–arsenite reaction using a Technicon AutoAnalyzer system[2]. In this method, however, sublimation of the excess iodine is time-consuming and some non-reversible adsorption of iodine molecules onto the plate occurs.

The studies reported here were undertaken to improve these disadvantages and to produce a simple and accurate method. The reaction of MDMs (or PMDMs) with iodine was conducted in the liquid phase and the amount of MDM (or PMDM)–iodine complexes was determined by their absorption at 375 nm.

EXPERIMENTAL

Materials and reagents

Authentic samples of MDMs and PMDMs were obtained using previously described methods[1,3*]. All reagents were of special grade and purchased from Wako (Osaka, Japan).

Apparatus

A Beckman DB-GT spectrophotometer and 1-cm quartz glass cells were used.

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* Structures of MDMs and PMDMs were shown in the previous paper[4].

Thin-layer chromatography

Adsorbent layer. Silica gel thin-layer plates were prepared by a method previously reported[2].

Solvent systems. Solvent A: the upper layer of a mixture of *n*-hexane–diethyl ether–isopropanol–water (1:4:1:2). Solvent B: chloroform–ethanol–28% ammonia water (50:1:0.1). Solvent C: the upper layer of a mixture of *n*-hexane–diisopropyl ether–ethanol–water (3:20:5:4).

Sample application and development. MDMs were separated by two-dimensional TLC using the ascending technique. The sample (25 mg) was dissolved in 1000 μl of chloroform, of which 80 μl was applied to the plate in a corner 2 cm from the bottom and left side edges. The first development was carried out with solvent A. After evaporation of the solvent (*ca.* 1 h at room temperature), the second development was done with solvent B in a direction lateral to the first.

PMDMs were separated by one-dimensional TLC. A chloroform solution of PMDMs (2 mg/80 μl) was applied to the plate at 2 cm above the bottom edge in the shape of a band, and the plate was developed with solvent C.

Extraction of the spots. After evaporation of the solvent, the plate was placed in an atmosphere of iodine vapour for *ca.* 1 min to visualize and delineate the spots. Each spot was scraped off with a spatula and transferred to a 10-ml test-tube. Methanol (5 ml) was added to each tube and the suspension was vigorously shaken for 10 min and then centrifuged at 1400 *g* for 5 min. A sample of the supernatant was pipetted into another test-tube and the methanol was evaporated by blowing with dry nitrogen gas.

Preparation of iodine complex

To the residue was added 5 ml of a $5 \cdot 10^{-3}$ *M* solution of iodine in 1,2-dichloroethane. The mixture was shaken for 10 min and allowed to stand at room temperature for 20 min, then absorbance at 375 nm was measured. The standard curves of MDM III and PMDM III were used for the determination of all the components of MDMs and PMDMs, respectively (Fig. 1).

RESULTS AND DISCUSSION

Thin-layer chromatography

Thin-layer chromatographic separation of PMDMs was carried out by a method previously reported[2]. In the case of MDMs, a more polar solvent had to be used for the development and the resulting spots were broad enough to necessitate the use of a two-dimensional method. Fig. 2 gives a typical chromatogram of MDMs.

Recoveries of MDM III and PMDM III from the silica gel plate were good when more than 50 μg of the compound was applied, *e.g.*, 100% for 70 μg and 90% for 50 μg, but 70% for 20 μg and 55% for 10 μg. Thus as much as 2000 μg had to be loaded on the TLC plate for a sample containing minor components. Since each upper limit of the linear calibration curves of MDM III and PMDM III was *ca.* 40 μg/ml, the volume of the sample solution pipetted out from the supernatant was adjusted according to the ratio of the components.

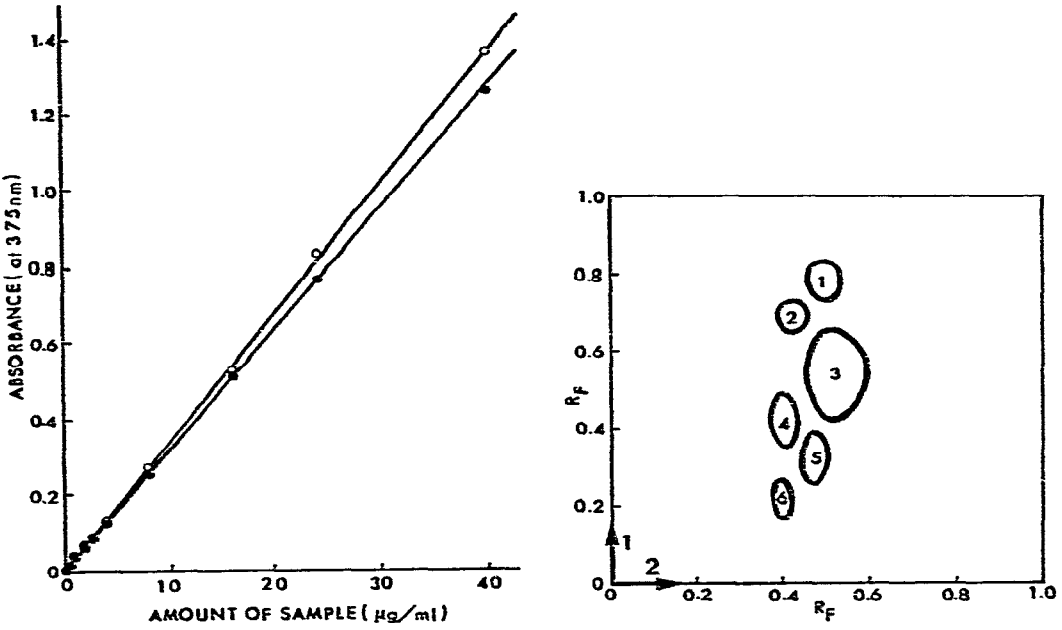

Fig. 1. Calibration curves of MDM III (○) and PMDM III (●).

Fig. 2. Typical thin-layer chromatogram of MDMs. Conditions: plate, Merck silica gel G Type 60; solvent, first development = upper layer of a mixture of *n*-hexane–diethyl ether–isopropanol–water (1:4:1:2), second development = chloroform–ethanol–28% ammonia water (50:1:0.1); detection, iodine vapour. Spots: 1 = MDM I; 2 = MDM II; 3 = MDM III; 4 = MDM IV; 5 = MDM V; 6 = MDM VI.

Reaction with iodine

When PMDM III (main component of PMDMs) was added to an iodine solution in 1,2-dichloroethane, absorption maxima were observed at 292 nm (ε = 38,000) and 375 nm (ε = 28,000) as shown in Fig. 3. Such absorption bands could be ascribed to the formation of a triiodide complex[5–7]. The reaction is considered to proceed as follows[8,9]:

$R\text{–}N(CH_3)_2 + I_2 \longrightarrow R\text{–}N(CH_3)_2I_2$	outer complex (1:1 charge-transfer complex)	(1)
$R\text{–}N(CH_3)_2I_2 \longrightarrow [R\text{–}N(CH_3)_2I]^+I^-$	inner complex	(2)
$[R\text{–}N(CH_3)_2I]^+I^- \longrightarrow [R\text{–}N(CH_3)_2I]^+ + I^-$	dissociation	(3)
$I^- + I_2 \longrightarrow I_3^-$	triiodide	(4)

The reaction was also observed in other dipolar aprotic solvents such as chloroform or dichloromethane. But 1,2-dichloroethane was chosen as the reaction solvent because of its relatively low volatility, high purity and provision of a stable reaction product.

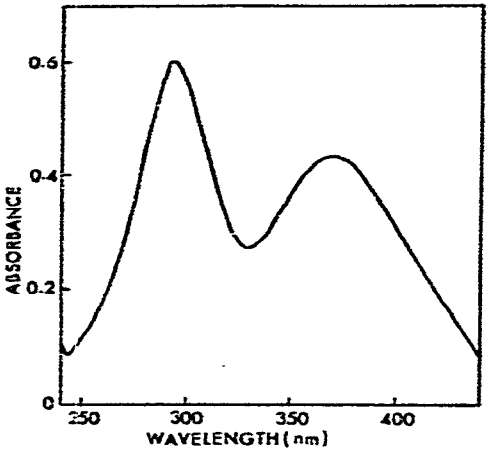

Fig. 3. Absorption spectra of the reaction mixture of PMDM III ($1.56 \cdot 10^{-5}$ *M*) and iodine ($5.0 \cdot 10^{-3}$ *M*) in 1,2-dichloroethane measured at 25° using the iodine solution as the reference.

In the case of protic solvents such as alcohols or aqueous solutions of alcohols, iodine was mostly complexed with the solvents rather than with PMDM III. In particular, PMDM III was converted into des-N-methyl PMDM III with the formation of formaldehyde in aqueous solutions of alcohols[10]. When a reaction of PMDM III with iodine was carried out in carbon tetrachloride, a non-polar solvent, absorption maxima were observed at 262 and 425 nm and agreed very closely with those obtained from the (1:1) trimethylamine iodine complex. The maxima were attributed to the charge-transfer band and blue-shifted visible band of the outer complex of PMDM III with iodine[11]. This indicated that further reaction corresponding to steps 2, 3 and 4 did not proceed in non-polar solvent. The absorbance at 262 nm was linearly related to the concentration of PMDM III up to *ca.* 50 μg/ml in 0.01 *M* iodine solution. However, this non-ionic charge-transfer complex is fairly unstable against moisture.

The reaction rate of triiodide formation in 1,2-dichloroethane was determined by the changes in the absorbance at 375 nm. The reaction was of the first order with respect to the concentration of PMDM III and iodine, the kinetic constant being 0.627 l mol^{-1} sec^{-1} at 25°. This result shows that the rate-limiting step of the reaction

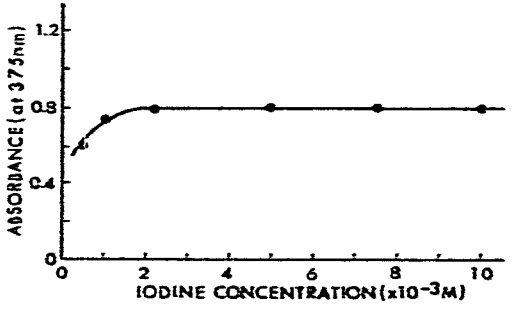

Fig. 4. Effect of iodine concentration. Conditions: sample, PMDM III, $2.88 \cdot 10^{-5}$ *M*; reaction time, 30 min; temperature, 25°.

processes is step 1, and the subsequent reactions 2–4, proceed quickly. In order to use this reaction for the ratio analysis of MDMs or PMDMs, the concentration of the iodine solution had to be more than $2 \cdot 10^{-3}$ *M* as shown in Fig. 4. Hence the iodine concentration and the reaction time were set at $5 \cdot 10^{-3}$ *M* and 30 min, respectively.

Using the method outlined, the artificial mixture of known amounts of MDM I, II, III, IV, V and VI was analysed and satisfactory results were obtained, as shown in Table I.

TABLE I

ANALYTICAL DATA OF A MODEL SAMPLE OF KNOWN COMPOSITION

	Composition of MDM (%)					
	I	*II*	*III*	*IV*	*V*	*VI*
Artificial mixture	2.0	3.0	83.0	7.0	4.0	1.0
Test 1	1.5	2.8	83.6	6.9	4.3	0.9
Test 2	1.7	3.5	82.6	7.0	4.4	0.7
Test 3	1.9	2.3	82.8	7.2	5.0	0.8
Test 4	1.8	2.8	82.8	7.2	4.8	0.6
Test 5	1.7	2.1	81.5	8.8	5.0	0.9
Mean	1.7	2.7	82.7	7.4	4.7	0.8

This method is applicable to other macrolide antibiotics which have a tertiary-bonded nitrogen structure in their molecule, such as leucomycins, carbomycins and erythromycins.

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