Journal of Chromatography, 169 (1979) 329–336

CHROM. 11,483

ANALYTICAL STUDIES OF MARIDOMYCIN

I. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MARIDOMYCINS AND SOME OTHER MACROLIDE ANTIBIOTICS

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(Received September 19th, 1978)

SUMMARY

Maridomycins and their acyl derivatives (9-propionyl, 2′-propionyl and 9,2′-dipropionyl maridomycins) were separated quantitatively into six components by high-performance liquid chromatography using a Corasil I column (200 cm × 2 mm I.D.) with a mixed sovent (upper layer of *n*-hexane, diisopropyl ether, ethanol and water) as the eluent. A linear relationship was found between the logarithm of the capacity factors (k') and the logarithm of the alkyl carbon numbers in the acyl group at position 4″. This relationship led to the discovery of a new component of maridomycin (3-propionyl-4″-*n*-butyryl analogue) in a crude sample. A similar relationship was found on chromatographic separation of some other macrolide antibiotics such as leucomycin, carbomycin A and B groups, under similar conditions.

INTRODUCTION

Maridomycin (MDM) is a new macrolide antibiotic which was obtained[1] from the culture medium of *Streptomyces hygroscopicus* No. B-5050. It consists of six components, namely MDM I, II, III, IV, V and VI[2], the structures of which differ only in the acyl groups at positions 3 and 4″ (Fig. 1). Thus, these components have similar characteristics. Some chemical modifications of MDM such as the acylation of hydroxyl groups at positions 9 and 2′ (ref. 3) were done to improve the medical availability. Although paper and thin-layer chromatography[4,5] have been used to separate these components, complete separation could not be attained. Recently, a method for separating leucomycin components was reported using high-performance liquid chromatography (HPLC) with a reversed-phase column[6]. However, it could not separate the components sufficiently for quantitative analysis. This paper deals with the separation of MDMs and their 9-propionyl derivatives (PMDMs) into their components using HPLC. Some analogous propionyl derivatives of MDMs (2′-propionyl and 9,2′-dipropionyl MDMs) were also separated into their components under the conditions used for PMDMs. The chromatographic system uses a silica

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	R_1	R_2
I	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$
II	$COCH_3$	$COCH_2CH(CH_3)_2$
III	$COCH_2CH_3$	$COCH_2CH_3$
IV	$COCH_3$	$COCH_2CH_3$
V	$COCH_2CH_3$	$COCH_3$
VI	$COCH_3$	$COCH_3$

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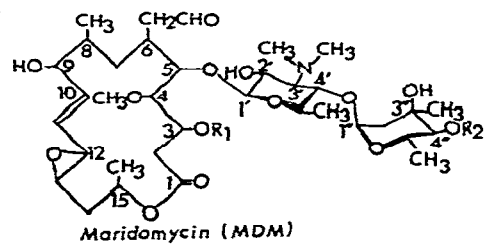
	R_1	R_2
YL-704A_1	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$
Leucomycin A_3	$COCH_3$	$COCH_2CH(CH_3)_2$
Leucomycin A_1	H	$COCH_2CH(CH_3)_2$
Leucomycin A_4	$COCH_3$	$COCH_2CH_2CH_3$
Leucomycin A_5	H	$COCH_2CH_2CH_3$
SF-837A_1	$COCH_2CH_3$	$COCH_2CH_3$
Leucomycin A_6	$COCH_3$	$COCH_2CH_3$
Leucomycin A_7	H	$COCH_2CH_3$
YL-704C_2	$COCH_2CH_3$	$COCH_3$
Leucomycin A_8	$COCH_3$	$COCH_3$
Leucomycin A_9	H	$COCH_3$



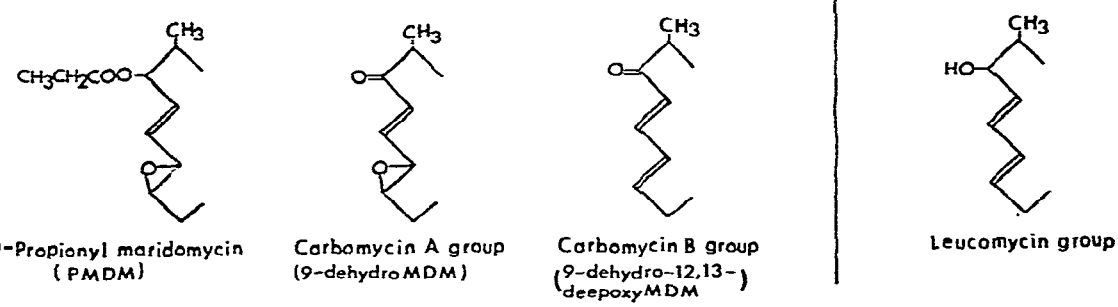


Fig. 1. Structural formulae of MDMs, their derivatives and other macrolide antibiotics.

gel column and the upper layer of a mixed solvent of *n*-hexane, diisopropyl ether, ethanol and water, to which a small amount of ethanol is added. This system has been used to separate polyether-polyols by the number of their hydroxyl groups (*i.e.*, polarity of the molecules)[7].

EXPERIMENTAL

Apparatus

An analytical liquid chromatograph ALC-201 (Waters Assoc., Milford, Mass., U.S.A.) equipped with a differential refractometer was used throughout the investigations, and a UV monitor (254 nm, Laboratory Data Control) was also used occasionally. The column was prepared by packing Corasil I (Waters Assoc.) into stainless-steel tubes (60 or 200 cm × 2 mm I.D.) using the dry packing technique. The length was changed in each experiment, and the temperature was controlled at 25 ± 1°. The samples were injected into the column through an ethylene–propylene rubber septum using a microsyringe (Termo Co.).

Elution solvent

Analytical-grade solvents were purchased from Wako (Osaka, Japan). A mixture of *n*-hexane–diisopropyl ether–ethanol–water (1:4:1:2 for MDMs or 5:20:4:10 for PMDMs) was shaken in a separatory funnel and allowed to stand overnight at 25 ± 1°. The upper layer was separated and 4% (for MDMs) or 3.5% (for PMDMs) of ethanol was added.

Samples

Authentic MDMs[2] and PMDMs[3] were obtained by the methods described in previous reports. Leucomycins[8] and SF-837A_1 (ref. 9) were purchased from Toyo Jozo (Shizuoka, Japan) and Meiji Seika (Tokyo, Japan), respectively. Carbomycin A group was obtained by chemical conversion of MDMs[10,11]. Carbomycin B group was obtained by de-epoxidation of the carbomycin A group[10]. YL-704A_1 (ref. 12) and C_2 (ref. 13) were derived from SF-837A_1 by the methods of Nakahama *et al.*[14], Muroi *et al.*[15], and Uchida *et al.*[16]. These samples together with internal standards (neopentylglycol for MDMs and testosterone for PMDMs) were dissolved in a small portion of the elution solvents. Structures of the samples are shown in Fig. 1.

RESULTS AND DISCUSSION

The adsorbability of silica gel generally changes with the quantity of water adsorbed on the surface; *i.e.*, it decreases as the amount of adsorbed water increases. On the other hand, the polarities of chromatographic solvents can be controlled by varying the mixing ratio of the organic solvents. Thus, the chromatographic system used in this study was a combination of a mixed organic solvent containing water (a mixture of *n*-hexane, diisopropyl ether, ethanol and water) and a silica gel column (Corasil I). Preliminary studies using a short column (60 cm) showed that the retention volumes of the peaks of MDM components gradually varied with the running time as shown in Fig. 2a. The upper layer of the equilibrated mixed solvent without any further addition of ethanol was used as the eluent. Thus, we presumed that a slight

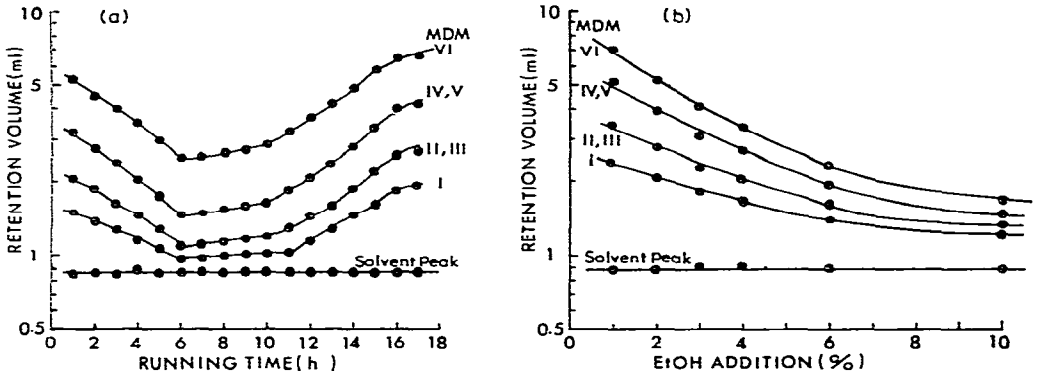


Fig. 2. (a) Variation in the retention volumes of MDMs in continual operation. Conditions: column, Corasil I (37–50 μm) 60 cm × 2 mm I.D. stainless steel; temperature, 25°; solvent, upper layer of a mixture of *n*-hexane–diisopropyl ether–ethanol–water (1:4:1:2); flow-rate, 0.35 ml/min; sample size, 10 μl (56 μg MDMs). (b) Effect of ethanol addition to the upper layer of the solvent mixture on the retention volume. Conditions: solvent, upper layer of a mixture of *n*-hexane–diisopropyl ether–ethanol–water (1:4:1:2) to which ethanol was added; other conditions are the same as those in (a).

change of column temperature under the experimental conditions would severely affect the equilibrium state of the solvent; *i.e.*, a change in column temperature would induce deposition of water from the solvent, and the water might occupy adsorption sites on the surface of the silica gel. Therefore, a small amount of ethanol was added to the elution solvent (upper layer) to prevent water deposition. In this case, the retention volumes of the peaks decreased with an increase in added ethanol, and the separations of MDM II and III and of IV and V did not improve (Fig. 2b). To separate these four components sufficiently, the column was lengthened from 60 to 200 cm. A typical chromatogram of the separation of MDMs is shown in Fig.

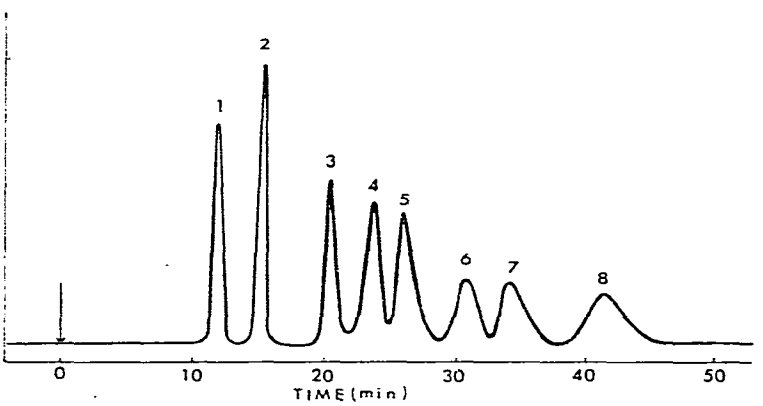


Fig. 3. Typical chromatogram of a test mixture of MDMs. Conditions: column, Corasil I (37–50 μm) 200 cm × 2 mm I.D. stainless steel; temperature, 25°; solvent, upper layer of a mixture of *n*-hexane–diisopropyl ether–ethanol–water (1:4:1:2) to which 4% of ethanol was added; sample size, 15 μl (100 μg); detector, refractometer (4×). Peaks: 1 = solvent; 2 = internal standard (neopentyl glycol); 3 = MDM I; 4 = MDM II; 5 = MDM III; 6 = MDM IV; 7 = MDM V; 8 = MDM VI.

3. The coefficient of variation calculated from the peak areas in nine individual experiments was about 1%.

Similarly, the conditions for separating PMDMs were established using the Corasil I column (200 cm × 2 mm I.D.). The eluent was the upper layer of a mixture of *n*-hexane–diisopropyl ether–ethanol–water (5:20:4:10), to which 3.5% of ethanol was added. Fig. 4 shows a typical chromatogram of the separation of PMDMs. Two analogous propionyl derivatives (2′-propionyl and 9,2′-dipropionyl MDMs) could be separated into their components under the same conditions.

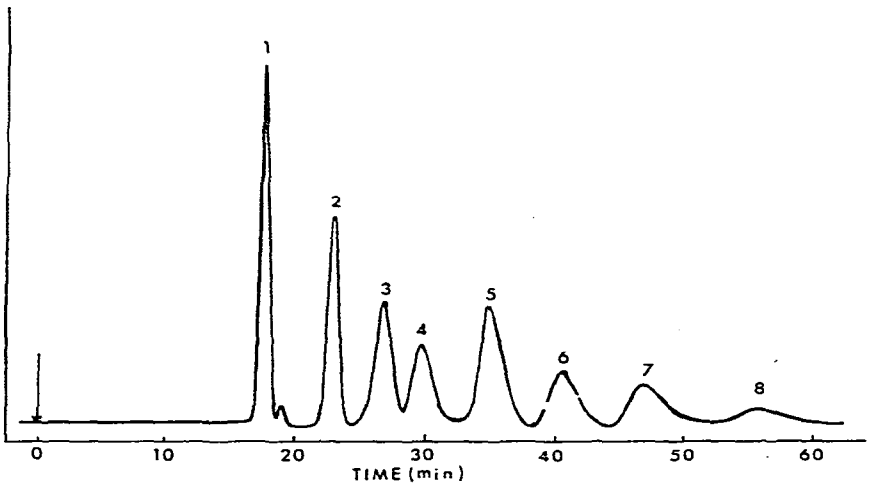


Fig. 4. Typical chromatogram of a test mixture of PMDMs. Conditions: solvent, upper layer of a mixture of *n*-hexane–diisopropyl ether–ethanol–water (5:20:4:10) to which 3.5% of ethanol was added; other conditions are the same as those in Fig. 3. Peaks: 1 = solvent peak; 2 = internal standard (testosterone); 3 = PMDM I; 4 = PMDM II; 5 = PMDM III; 6 = PMDM IV; 7 = PMDM V; 8 = PMDM VI.

The retention time of each peak under these experimental conditions was constant even when the sample amount was varied between 5 and 130 μg. As the relative responses of these components to the refractometer were practically the same, the calibration curves of MDM III or PMDM III were used to quantitate MDMs or PMDMs. The calibration curves in Fig. 5 were calculated from the peak areas and corrected for the sensitivity of each internal standard.

The chromatograms of MDMs or PMDMs showed that the logarithm of the capacity factors (k'), which were calculated from the retention volume of each peak, is linearly related to the logarithm of the number of alkyl carbons in the acyl group at position 4″, and the slopes of these linear rates for 3-acetyl compounds and 3-propionyl compounds in every group are parallel as shown in Fig. 6. These facts indicate the prospective retention volumes of other derivatives which have different numbers of alkyl carbons at their 4″-position. In fact, the small unknown peak between MDM I and II, which is sometimes found on chromatograms of crude samples, was anticipated to include three carbons at position 4″ from the log k' value. Preparative chromatography and physicochemical determinations showed that it was 3-propionyl-4″-*n*-butyryl analogue (a new component)[17].

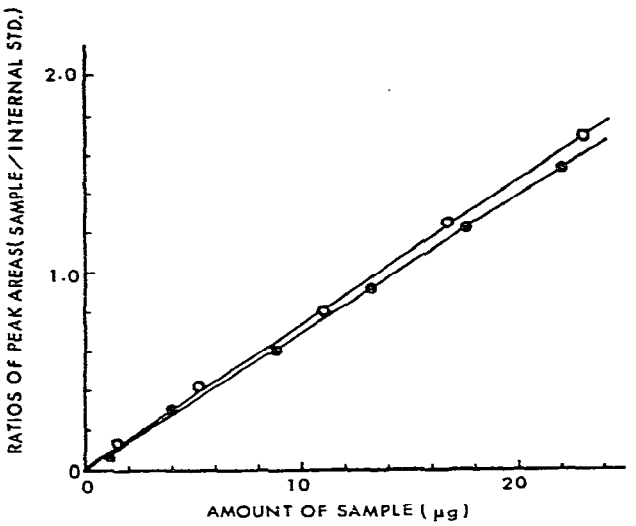


Fig. 5. Calibration curves of MDM III (○) and PMDM III (●). Conditions: the same as those in Figs. 3 and 4, respectively. Internal standard: neopentyl glycol (14.4 μg) for NDM III and testosterone (7.5 μg) for PMDM III.

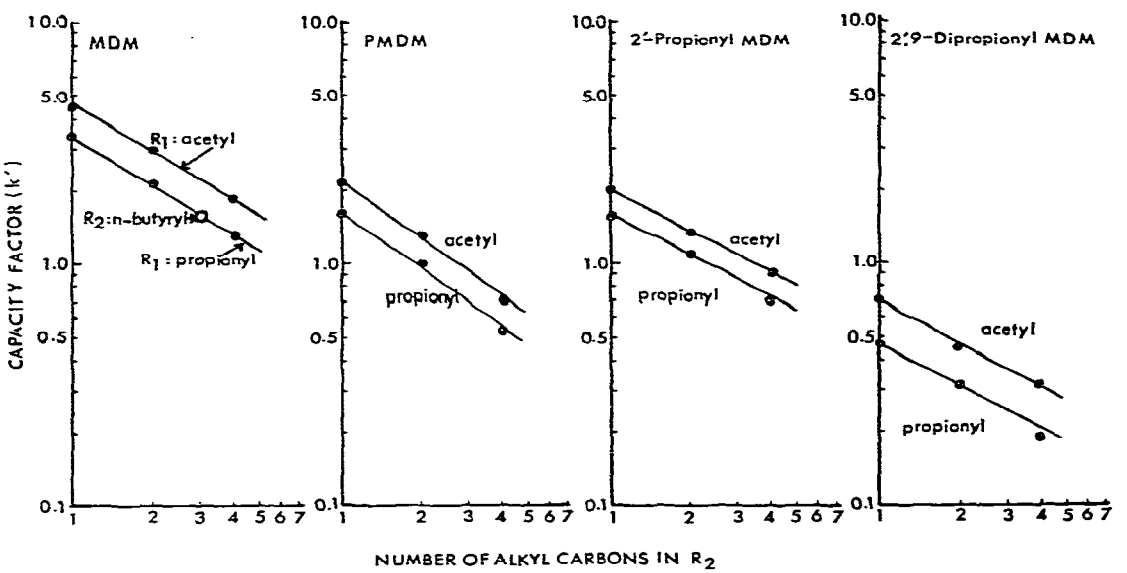


Fig. 6. Linear relationships between the logarithms of capacity factors and the numbers of alkyl carbons in the acyl group at position 4″ of MDMs and their propionyl derivatives.

The linear relationship between the log k' and the log of the number of alkyl carbons in the 4″-acyl group is

$$\log k' = A \log N + B \tag{1}$$

where A and B are constant under the experimental conditions and N is the number of alkyl carbons in the 4″-acyl group. Kuchař *et al.*[18] have conducted experiments

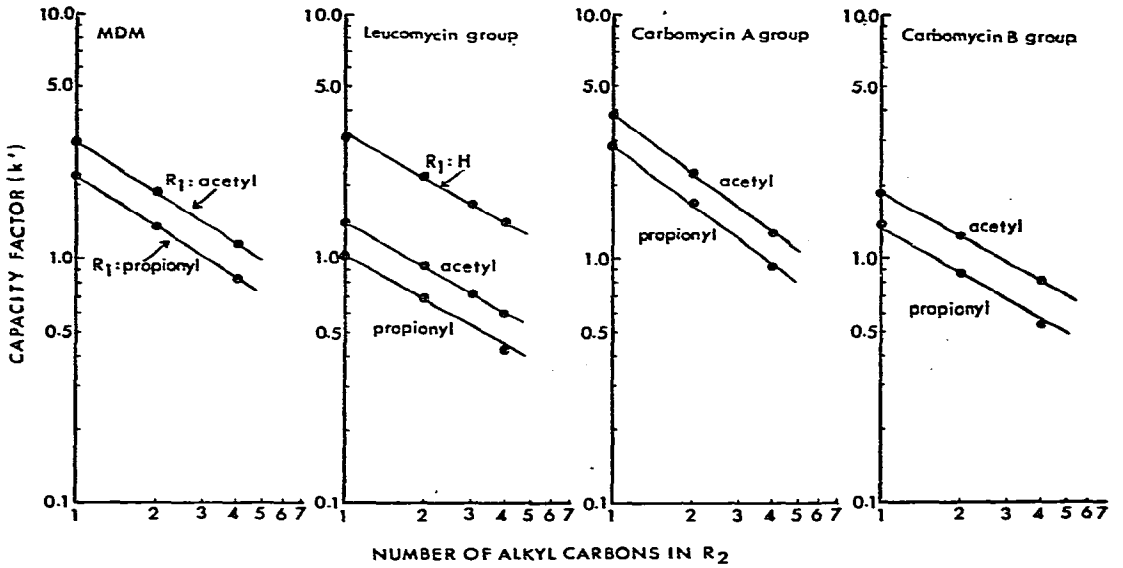


Fig. 7. Linear relationships between the logarithms of capacity factors and the number of alkyl carbons in the acyl group at position 4″ of some sixteen-membered macrolide antibiotics.

TABLE I

SEPARATION FACTOR OF MACROLIDE ANTIBIOTICS WHEN THE ACYL GROUP AT POSITION 3 IS ACETYL OR PROPIONYL

Conditions: A, same as those in Fig. 3; B, same as those in Fig. 4.

Macrolide antibiotic	*Acyl group at position 4″ (R_2)*	$k'_{COCH_3}/k'_{COCH_2CH_3}$	*Conditions*
Leucomycin group	$COCH_3$	1.34	A
	$COCH_2CH_3$	1.35	
	$COCH_2CH(CH_3)_2$	1.44	
MDM	$COCH_3$	1.32	A
	$COCH_2CH_3$	1.38	
	$COCH_2CH(CH_3)_2$	1.37	
Carbomycin A group	$COCH_3$	1.30	A
	$COCH_2CH_3$	1.33	
	$COCH_2CH(CH_3)_2$	1.36	
Carbomycin B group	$COCH_3$	1.36	A
	$COCH_2CH_3$	1.38	
	$COCH_2CH(CH_3)_2$	1.45	
4″-Deacyl MDM	H	1.33	B
MDM	$COCH_3$	1.34	B
	$COCH_2CH_3$	1.39	
	$COCH_2CH(CH_3)_2$	1.44	
PMDM	$COCH_3$	1.30	B
	$COCH_2CH_3$	1.31	
	$COCH_2CH(CH_3)_2$	1.35	
2′-Propionyl MDM	$COCH_3$	1.31	B
	$COCH_2CH_3$	1.23	
	$COCH_2CH(CH_3)_2$	1.34	
2′,9-Dipropionyl MDM	$COCH_3$	1.48	B
	$COCH_2CH_3$	1.41	
	$COCH_2CH(CH_3)_2$	1.36	

using paper chromatography and reported that the relationship between the migration ratio (R_M) and the partition coefficients (D) is

$$R_M = a \log D + b = \log \left(\frac{1}{R_F} - 1 \right) \quad (2)$$

where a and b are constant under the experimental conditions used. As the R_M value is thought to be analogous to $\log k'$, the two above equations are comparable and the number of alkyl carbons in the 4″-acyl group should directly affect partition coefficients under this chromatographic condition. This linear relationship was also found in the chromatograms of other macrolide antibiotics such as leucomycin group, carbomycin A group and carbomycin B group (Fig. 7).

On the other hand, the difference in the number of alkyl carbons in the acyl group at position 3 (acetyl or propionyl) directly affects the separation factor ($\alpha = k'_1/k'_2$). The ratios of $k'_{COCH_3}/k'_{COCH_2CH_3}$ in the investigated samples were practically the same as those in Table I, even if the structures of the sixteen-membered ring were different. This may assist in identifying unknown peaks.

ACKNOWLEDGEMENT

The author wishes to thank Dr. E. Ohmura, Director of this Division and Dr. M. Nishikawa, Director of these Laboratories, for the continuing interest and encouragement. I also thank Drs. T. Kishi and M. Hori for their helpful advice and discussions.

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