Radioimmunoassay for Metabolites of 9,3'-Diacetylmidecamycin, a Macrolide Antibiotic

NORIAKI SHIMADA,1* TAKAO UEDA,1 TETSUYOSHI YOKOSHIMA,1 KOSHIRO UMEMURA,* AND TOMOKO SHOMURA2

Tokai Laboratory, Daiichi Pure Chemicals Co. Ltd., 2,117 Tokai-mura, Ibaraki 319-11,1 and Central Research Laboratories, Meiji Seika Kaisha Ltd., Kohoku-ku, Yokohama, Kanagawa 222,2 Japan

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A radioimmunoassay system has been developed for the measurement of two major metabolites of 9,3'-diacetylmidecamycin, Mb-6 and Mb-12. A radioimmunoassay for Mb-6 was performed by using anti-Mb-6 serum and a [125I]tyramined Mb-6 derivative as a radiolabeled antigen. The labeled antigen was prepared by the chloramine T method. The antiserum was obtained from a rabbit immunized with Mb-6 conjugated to bovine serum albumin. The obtained antiserum was cross-reactive with two other metabolites of 9,3'-diacetylmidecamycin, Mb-2 and Mb-12, in addition to Mb-6. This Mb-6 radioimmunoassay system could detect Mb-6 concentrations as low as 100 pg/ml of serum. The coefficients of variation were 4.5% (intra-assay) and 5.1% (inter-assay). A radioimmunoassay for Mb-12, using anti-midecamycin serum and a [125I]tyramined-Mb-12 derivative, has also been developed. The antiserum was cross-reactive only with Mb-12 among the 9,3'-diacetylmidecamycin metabolites. This Mb-12 radioimmunoassay system could detect Mb-12 concentrations as low as 2 ng/ml. The intra- and inter-assay variances were 5.9 and 5.8%, respectively.

9,3'-Diacetylmidecamycin (MOM) is a derivative of a macrolide antibiotic, midecamycin (MDM). These macrolide antibiotics exhibit a potent antibacterial action against gram-positive bacteria and mycoplasmas (3; Y. Kazuno, K. Miyauchi, Y. Origasa, T. Yoshida, T. Ishii, T. Watanabe, R. Okamoto, and M. Kiryu, Abstr. 27th Jpn. Cong. Chemother., p. 11, 1979). MOM has a higher potency and lower toxicity than other macrolides; its therapeutic effects are observed at doses half those of other macrolides. It has been reported that MOM administered orally to humans is rapidly metabolized to Mb-1 before it reaches the liver, and this product is then immediately metabolized through two metabolic pathways (1, 4). The major metabolic pathway is from Mb-1 to Mb-9a via Mb-12 and Mb-6, and the minor one is from Mb-1 to Mb-9a or Mb-9b via Mb-2, Mb-3, and Mb-5. The major metabolites in blood or urine are Mb-6, Mb-12, and Mb-9a. Neither unchanged MOM nor Mb-1 is detectable in either blood or urine by chemical assays using thin-layer chromatography (1). MOM metabolites have more or less antimicrobial activity. The activity of Mb-12, estimated by antimicrobial assay in vitro, is almost the same as that of MOM (5). The structures of MOM and its metabolites, as well as of MDM, are given in Fig. 1.

To examine the relationship between the efficacy of MOM and the blood level of MOM metabolites, a sensitive assay system for MOM metabolites is required. Macrolide antibiotics have been measured by antimicrobial assay or chemical assay (1, 4–6); however, these methods are unsuitable for the routine assay for MOM metabolites because they are insensitive and time-consuming for the treatment of a large number of samples. In the present study, we aimed to establish a radioimmunoassay (RIA) system for the major metabolites of MOM. RIA procedures and the results obtained in a blood level study using the developed RIA system are described.

MATERIALS AND METHODS

Chemicals. Na125I (carrier free) was purchased from New England Nuclear Corp. Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. Freund complete adjuvant was obtained from Calbiochem-Behring Co. MDM, MOM, and MDM metabolites were supplied by Meiji Seika Kaisha Ltd. All other chemicals used in the present study were reagent grade.

Preparation of antigen. Mb-6 and MDM were cou-
Preparation of tracer. Mb-6 2'-hemisuccinate (42 mg), tyramine hydrochloride (13 mg), and \( N \) -hydroxybenzotriazole (10 mg) were dissolved in 1 ml of dimethylformamide. To this solution was added 12 mg of \( N,N' \)-dicyclohexylcarbodiimide. After stirring overnight at room temperature, the resulting conjugate (termed tyramine-suc-Mb-6) was separated by thin-layer chromatography with chloroform-methanol (5:1, vol/vol) as the solvent system. The obtained tyramine-suc-Mb-6 was radiolabeled with \(^{125}\text{I}\) by using chloramine T by the method of Hunter and Greenwood (2). The radiolabeled antigen was purified by gel filtration on Sephadex G-10, using 1 M acetic acid as the eluent.

Mb-12 2'-hemisuccinate was separated from a mixture of Mb-12 (16 mg) and succinic anhydride (3 mg) in 0.3 ml of anhydrous pyridine by using SiO\(_2\) column chromatography; the yield was 13 mg. To the hemisuccinate (10 mg), tyramine hydrochloride (4 mg), and \( N \) -hydroxybenzotriazole (3 mg), dissolved in 0.5 ml of dimethylformamide, was added 2 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; the mixture was then stirred overnight at room temperature. Tyramine-suc-Mb-12 was separated from the resulting reaction mixture by thin-layer chromatography with chloroform-methanol (9:1, vol/vol) as the solvent system; the yield was 0.73 mg. The obtained tyramine-suc-Mb-12 was radiolabeled with \(^{125}\text{I}\) by the chloramine T method and purified by gel filtration as described above.

**Immunization.** Mb-6-suc-BSA conjugate or MDM-suc-BSA conjugate was dissolved in 0.5 ml of 0.9% NaCl. To this solution was added 0.5 ml of Freund complete adjuvant, and the mixture was emulsified by a homogenizer. The emulsion was administered to seven female Japanese white rabbits intradermally at multiple sites five times every 2 weeks and then subcutaneously once a month for 3 months. The rabbits were bled 10 days after the last immunization. Antisera against Mb-6 and MDM which showed the highest titer were used in the present RIA system.

**RIA procedures.** A diluent for reagents was 10 mM sodium phosphate buffer containing 0.5% BSA, 25 mM EDTA, and 0.9% NaCl, pH 7.4 (PBS-BSA). The reaction mixture contained the following reagents in a plastic tube: 0.4 ml of PBS-BSA, 0.1 ml of standard or sample solution, 0.1 ml of 20-fold-diluted antisera (anti-Mb-6 serum or anti-MDM serum), and 0.1 ml of tracer \((1^{25}\text{I})\)tyramine-suc-Mb-6 or \((1^{25}\text{I})\)tyramine-suc-Mb-12, about 10,000 cpm. Tubes prepared in duplicate were incubated overnight at 4°C. Bound and free antigens were separated by using dextran-coated charcoal as follows. To each tube, 1 ml of sodium phosphate buffer containing 1 mg of dextran T-70 and 10 mg of charcoal (pH 7.4) was added. The tubes were incubated at 4°C for 30 min and then centrifuged at 1,600 \(\times \) g for 15 min at 4°C. The radioactivity of the supernatant fluid was determined in an Aloka Auto-gamma counter.

Specific binding was expressed as the difference of radioactivity between the presence and absence of tracer. The specific binding was 3,000 to 4,000 cpm in RIAs using anti-Mb-6 serum and anti-MDM serum.

**RESULTS**

**Specificity.** To test the specificity of anti-Mb-6 serum and anti-MDM serum for MOM metabolites such as Mb-2, Mb-3, Mb-5, Mb-6, Mb-9a, Mb-9b, and Mb-12, cross-reactivity studies were conducted (Fig. 2).
Cross-reactivity was expressed as the relative potency of each metabolite, compared to that of Mb-6, for 50% displacement of labeled Mb-6. The percentages of cross-reactivity of anti-Mb-6 serum for Mb-2 and Mb-12 were 84 and 202%, respectively (Table 1). Meanwhile, when cross-reactivity of anti-MDM serum was expressed as the relative potency of each metabolite, compared to Mb-12, for 50% displacement of labeled Mb-12, the percentage of cross-reactivity was less than 8% for all metabolites of MOM (Table 1).

**Recovery test.** Recovery experiments were performed by adding three different amounts of Mb-6 or Mb-12, which allowed about 70, 50, and 30% displacement of the binding of labeled antigen to normal human serum and assaying them by the RIA system. The average of 10 replicates at each concentration of Mb-6 was 84.9, 92.3, and 98.4%, respectively; the mean recovery was

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**FIG. 2.** Dose-response curves of MOM metabolites prepared in PBS-BSA in the (A) Mb-6 and (B) Mb-12 RIA systems. □, Mb-2; △, Mb-3; △, Mb-5; ●, Mb-6; ○, Mb-9a; ■, Mb-9b; O, Mb-12; □, MDM.
TABLE 1. Cross-reactivity of MOM metabolites in the RIA

<table>
<thead>
<tr>
<th>Assay for Metabolite</th>
<th>Cross-reactivitya (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mb-6 Mb-6</td>
<td>100</td>
</tr>
<tr>
<td>Mb-6 Mb-2</td>
<td>84</td>
</tr>
<tr>
<td>Mb-6 Mb-3</td>
<td>2</td>
</tr>
<tr>
<td>Mb-6 Mb-5</td>
<td>3</td>
</tr>
<tr>
<td>Mb-6 Mb-9a</td>
<td>8</td>
</tr>
<tr>
<td>Mb-6 Mb-9b</td>
<td>2</td>
</tr>
<tr>
<td>Mb-6 Mb-12</td>
<td>202</td>
</tr>
<tr>
<td>Mb-12 Mb-12</td>
<td>100</td>
</tr>
<tr>
<td>Mb-12 Mb-2</td>
<td>8</td>
</tr>
<tr>
<td>Mb-12 Mb-3</td>
<td>1</td>
</tr>
<tr>
<td>Mb-12 Mb-5</td>
<td>0</td>
</tr>
<tr>
<td>Mb-12 Mb-6</td>
<td>2</td>
</tr>
<tr>
<td>Mb-12 Mb-9a</td>
<td>5</td>
</tr>
<tr>
<td>Mb-12 Mb-9b</td>
<td>4</td>
</tr>
</tbody>
</table>

a As determined by the relative amounts needed for 50% displacement of radioactive antigen.

91.9%. Similarly, in RIA for Mb-12, the percentage of recovery at each concentration was 125.0, 106.1, and 96.0%, respectively, and the mean recovery was 109.0%.

Reproducibility. The reproducibility in the RIAs for Mb-6 and Mb-12 was estimated from the coefficient of variation (CV) in intra-assay and inter-assay systems. To determine inter- and intra-assay CVs, 10 replicates of metabolite-containing sera were measured in the same assay at three different concentrations (Table 2).

Serum Mb-6 and Mb-12 levels. The concentrations of Mb-6 equivalent and Mb-12 in the sera of patients orally administered 200 mg of MOM were measured by RIA. The typical patterns of serum levels of these metabolites are shown in Fig. 3. The concentration of Mb-6 equivalent in the serum was about threefold that of Mb-12.

DISCUSSION

The major metabolites of MOM in humans are Mb-6, Mb-12, and Mb-9a in blood and Mb-6 and Mb-12 in urine (1). In the present study, we attempted to develop an RIA system to assay Mb-6 and Mb-12 in biological fluids.

In the present RIA systems, anti-Mb-6 serum was cross-reactive with two other MOM metabolites, Mb-12 and Mb-2, whereas anti-MDM serum was cross-reactive only with Mb-12. Therefore, the RIA system using anti-MDM serum is effective for the determination of Mb-12 because MDM is not found in MOM metabolites (1). Sufficient amounts of Mb-12 to immunize rabbits could not be obtained; however, the structure of MDM is similar to that of Mb-12 except for the 4'-position (MDM, 4'-propionyl; Mb-12, 4'-acetyl), so MDM conjugated to BSA was used as the antigen to obtain the antisera used in the RIA for Mb-12. It has been reported that the Mb-2 concentration in human blood or urine is slight (1). Therefore, the Mb-6 equivalent concentration in biological fluids, obtained by the present RIA, was supposed to be the sum of Mb-6 and Mb-12. The reason why anti-Mb-6 serum exhibited higher affinity to Mb-12 than to Mb-6 is unclear.

The sensitivities of the present RIA system, expressed as the response equal to twice the standard deviation of a duplicate assay without unlabeled Mb-6 or Mb-12 were 0.1 ng/ml of serum in the RIA for Mb-6 and 2 ng/ml of serum in the RIA for Mb-12. These sensitivities are enough to determine concentrations of these MOM metabolites in the blood of a human administered the therapeutic dose of MOM. The sensitivities of both RIA systems were slightly lower in the determination of these metabolites.

FIG. 3. Mb-12 (○) and Mb-6 equivalent (●) levels in patient serum after oral administration of MOM (200 mg per patient).
in urine or buffer. However, high concentrations of these metabolites are ordinarily present in urine, so the lower sensitivity is not a practical problem.

Macrolide antibiotics have been measured by antimicrobial assay or chemical assay systems. Metabolites of these antibiotics cannot be measured separately by antimicrobial assay, but can be detected by chemical assay using thin-layer chromatography. However, the sensitivity of both chemical and antimicrobial assays is less than that of RIA. The RIA systems described in the present paper, therefore, appear to be suitable for the sensitive measurement of metabolites of MOM.

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LITERATURE CITED


