Radioimmunoassay for Clindamycin

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We describe a radioimmunoassay for measuring clindamycin in serum extracts that is more accurate than the microbiological assay because of the minimal response of the metabolite, *N*-demethylclindamycin. The assay is not affected by the presence of other antibiotics. As described it is as sensitive as the microbiological assay, but can be made more sensitive.

Additional Keyphrases: antibiotics in serum and urine • comparison with microbiological assay • drug assay

Clindamycin is a semisynthetic antibiotic in which a 7(S)-chloro group is substituted for the 7(R)-hydroxyl group on the parent compound, lincomycin (1). We needed to measure more accurately the concentrations of clindamycin in serum, both alone and in the presence of other antibiotics. Accordingly, we developed the radioimmunoassay reported here.

Materials and Methods

Preparative Methods

[**N**-methyl-3H]clindamycin-HCl·CH₃CH₂OH. *N*-Demethylclindamycin-HCl, 100 mg (0.22 mmol), was dissolved in 5 ml of water. The solution was cooled to 5 °C in an ice bath. Formaldehyde (370 g/liter, in water) 40 μl (0.53 mmol) was added. After 15 min, most of the *N*-demethylclindamycin was converted to the reactive intermediate, as shown by thin-layer chromatography (TLC) on silica gel with ethylacetate, acetone, and water (8/5/1 by vol) as developing solvent. Sodium borohydride, 10 mg (0.26 mmol), equivalent to 1.04 mmol of H₂, was added. Hydrogen evolution began immediately, and the product began to separate as an oil. After 75 min, the reaction was complete as shown by the previously used TLC system. The basic reaction mixture was extracted with CHCl₃ (5 × 10 ml). The CHCl₃ solution was dehydrated with Na₂SO₄ and evaporated to about 3 ml. The CHCl₃ solution was applied to a 20-mm (i.d.) column of dry-packed silica gel, 50 g, which had been stirred with a 10/1 by volume mixture of CHCl₃/methanol, 80 g being added per kilogram of gel. The column was eluted with the same solvent and 10-ml fractions were collected. The product was located, by TLC of effluent fractions, in tubes 9 and 10.

The contents of tubes 9 and 10 were combined and the solvent was evaporated. The residue was dissolved in 0.5 ml absolute ethanol, and three drops of ethanolic HCl were added. Crystallization occurred on cooling. The product was filtered and washed with ethylacetate/ethanol, 10/1 by volume. The yield was 61.9 mg, 57.3%, mp 156–160 °C (dec.). The infrared spectra was identical with that from an authentic sample. The final labeling was done by Mallinckrodt/Nuclear, St. Louis, Mo. 63160, using this procedure. Figure 1 shows the reaction.

*Clindamycin-2-hemisuccinate.* Clindamycin-2-hemisuccinate was synthesized by the method of Sinkula et al. (2). The workup after the acid hydrolysis is a modification of that supplied by W. Morozowich (3). 3,4-O-p-Anisylideneclindamycin-HCl, 5.0 g (8.6 mmol), and succinic anhydride, 5.0 g (50 mmol), were added to 50 ml of pyridine in a 500-ml flask fitted with a condenser and drying tube. The mixture was heated for 2.5 h at about 90 °C. After cooling, diethyl ether (500 ml) was added to precipitate the product as a gum. The precipitate was dissolved in 75 ml of glacial acetic acid. Water, 10 ml, was added and the mixture was heated for

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*Fig. 1. Synthesis of [**N**-methyl-3H]clindamycin*
45 min at about 90 °C. The solvent was evaporated and the residue dissolved in 25 ml of CHCl₃, and the solution seeded with succinic acid. The succinic acid was filtered off and washed with cold CHCl₃. Ether was added to the combined CHCl₃ extracts to precipitate the product, which was then dissolved in water and the pH adjusted to 6.0 with NH₄OH. The product crystallized from this mixture. It was recrystallized by dissolving in the minimum amount of CHCl₃ followed by the addition of hexane until crystals appeared (yield: 1.02 g, 22%; mp 169–172 °C). The infrared and nuclear magnetic resonance spectra were identical to those for an authentic sample obtained from W. Morozowich.

Clindamycin-2-hemisuccinyl/bovine serum albumin. Bovine serum albumin, 100 mg, was dissolved in 4 ml of water. The pH was adjusted to 5. Clindamycin-2-hemisuccinate, 100 mg, was added to 4 ml of water and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl, 100 mg, was added. The clindamycin-2-hemisuccinate mixture was added to the bovine serum albumin solution, the pH adjusted to 5, and the mixture allowed to remain at room temperature overnight. The mixture was dialyzed against distilled water and freeze dried. The yield was 116 mg. This conjugation procedure is shown in Figure 2.

The number of derivatized lysines attached to each bovine serum albumin molecule as determined by the trinitrobenzene sulfonic acid method (4) was 30.

Induction of antiserum. Two rabbits were injected intradermally with 10.0 mg of conjugate in an equimolar 50/50 mixture of saline (9 g of NaCl per liter) and Freund complete adjuvant (Difco Laboratories, Detroit, Mich. 48232). This was followed by 0.5 ml of pertussis vaccine (Eli Lilly & Co., Indianapolis, Ind. 46206). The rabbits then received monthly injections of 100 µg of conjugate in the saline/incomplete adjuvant mixture. After seven months, titers of 1/150 or greater were induced in both rabbits.

Sample preparation. Add 0.5 ml of serum to 2.5 ml of CHCl₃ and mix for 30 s. Centrifuge at 2000 rpm for 5 min, and aspirate the serum layer. Transfer an 0.1-ml aliquot of the CHCl₃ extract to a 1.5 × 5-cm polyethylene vial and evaporate under nitrogen. The sample is now ready for assay.

This extraction recovered an average of 90.4% of [³H]clindamycin added with the same amounts of unlabeled clindamycin as reported in the recovery and precision studies.

Standards. Dissolve clindamycin-HCl-H₂O in distilled water. The range is 1 to 250 ng/0.1 ml (10 to 2500 µg/liter).

Assay Procedure

Add 0.5 ml of phosphate buffer (50 mmol/liter phosphate, 9 g/liter NaCl, and 0.1 g/liter Na₂HPO₄; pH 8) to the residue in the sample vials to dissolve the antibiotic. Add an identical volume to the total-bound count vials. Only 0.4 ml of buffer is added to the standard vials, followed by 0.1 ml of standard solution. Add 0.1 ml of [³H]clindamycin (~1800 cpm) to all vials. To the mixture of labeled and unlabeled antibiotic in all vials add 0.2 ml of first antiserum diluted to bind 65–70% of label. Mix contents of all vials and allow to stand at room temperature for 2 h. After this initial incubation, add 0.2 ml of second antiserum diluted to bind all of first antibody. Mix and allow to stand overnight at 4 °C. After the second incubation, add 2.5 ml of buffer to each vial and centrifuge at 3000 rpm for 20 min. Decant the vials and allow to drain for 1 min. To each vial add 6 ml of scintillation cocktail containing 7570 ml of toluene, 250 ml of “Bio-Solv BBS-3” (Beckman Instruments, Fullerton, Calif. 92634) and 318 ml of “Liquifluor” (New England Nuclear, Boston, Mass. 02118). Mix to disperse the precipitated antibody–antibiotic complex. Count in a liquid scintillation counter until 500 counts have accumulated for the highest standard. If the samples contain concentrations of antibiotic greater than those in the highest standard, they can be assayed by taking a smaller aliquot of CHCl₃ extract or by diluting the serum before extraction. Repeated checking by means of the automatic, external-standard–channels ratio method showed little variation in quench when extracts were used as samples. The tritium counting efficiency was about 45%. Figure 3 shows an example of a standard curve in the log-logit transform.

Results

Analytical Variables

Cross-reactivity. The antiserum had a large cross-reactivity with clindamycin-2-phosphate (171%) and low cross-reactivity with clindamycin-2-palmitate (24%). This was the reverse of what was expected on the basis of structural similarity to clindamycin-2-emi-
sucinate. A possible explanation is that the long palmitate side-chain hydrophobically bonds to another portion of the clindamycin molecule, making it unrecognizable by the antibodies. This is not possible for the smaller, charged phosphate molecule. The phosphate group must satisfactorily mimic the carboxyl group of the hemisuccinate to render it more tightly bound than clindamycin.

The metabolite, clindamycin sulfoxide, cross-reacts 41.8%. This cross-reactivity is not desirable because this compound is a major urinary metabolite and a minor serum metabolite. A new conjugate has been prepared with freshly recrystallized hemisuccinate in an attempt to induce antisera without this problem.

Only lincomycin, of the other antibiotics tested, cross-reacted to a measurable degree (2.4%).

None of the components of the antibiotic or the common sugars cross-reacted. The results are shown in Table 1.

Recovery and precision studies. Recovery studies were on sera supplemented with five different concentrations of drug, from 5.00 to 0.100 mg/liter. Each value was measured four times in a single day for each of five days. The percent deviation and day-to-day CV are shown in Table 2. The average within-day CV for 20 determinations on two serum samples, containing ~1 and ~2 mg/liter, was 8.9%.

Sensitivity. The sensitivity of this procedure for clindamycin in serum is about 0.1 mg/liter, about the same as for the microbiological assay. However, the sensitivity of this assay depends primarily on the specific activity of the [3H]clindamycin, so this could be readily increased. Sodium [3H]borohydride is now available with specific activities of 15–20 kCi/mol. Synthesis with this borohydride could change the sensitivity to 5.0 μg/liter.

Comparison with Microbiological Assay (5)

Our assay generally produces values that are lower than those found with the microbiological assay. The difference tends to increase as the time after adminis-

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**Table 1. Cross-Reactivities**

| Clindamycin derivatives | & 171.0% |
| Clindamycin-2-phosphate | & 2.4% |
| Clindamycin-2-palmitate | & 8.1% |
| N-[3-Hydroxyethyl]clindamycin | & 8.1% |

**Table 2. Recovery and Precision**

<table>
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<th>Found</th>
<th>Added</th>
<th>Deviation, %</th>
<th>CV, %</th>
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<td>mg/liter</td>
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<tr>
<td>4.90 ± 0.23</td>
<td>5.00</td>
<td>- 2.0</td>
<td>4.69</td>
</tr>
<tr>
<td>2.00 ± 0.15</td>
<td>2.00</td>
<td>0.0</td>
<td>7.50</td>
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<tr>
<td>1.03 ± 0.13</td>
<td>1.00</td>
<td>+ 3.0</td>
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<tr>
<td>0.48 ± 0.068</td>
<td>0.50</td>
<td>- 4.0</td>
<td>14.2</td>
</tr>
<tr>
<td>0.11 ± 0.017</td>
<td>0.10</td>
<td>+ 10.0</td>
<td>15.4</td>
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</tbody>
</table>

*Mean of 20 determinations (±SD)*

**Table 3. Comparison of Results of Present Method with Those of Microbiological Assay, in Two Patients**

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Patient 3 Microbiol.</th>
<th>Patient 3 RIA</th>
<th>Patient 6 Microbiol.</th>
<th>Patient 6 RIA</th>
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<tr>
<td>12.0</td>
<td>0.82</td>
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<td>0.59</td>
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<tr>
<td>18.0</td>
<td>0.90</td>
<td>0.64</td>
<td>1.31</td>
<td>0.54</td>
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</table>

*After a 150-mg oral dose*
tation of the drug increases. The difference can be attributed to two factors. The microbiological assay is more sensitive to N-demethylclindamycin than to clindamycin (6). Brodasky et al. (7) have shown that when both N-demethylclindamycin and clindamycin are measured in serum at 1 and 8 h, the amount of N-demethyl averages 2.7% and 21% of the total, respectively. Therefore, N-demethyl makes a larger contribution to the microbiological assay as time after administration of drug increases. Except in patients who produce high concentrations of sulfoxide in the serum, this should be a more accurate assay than the microbiological one. In urine, with the high concentrations of sulfoxide, the microbiological assay would be more accurate.

Results for serum, as measured by both methods for two subjects after receiving a 150-mg oral dose of clindamycin-HCl, are shown in Table 3.

Discussion

Clindamycin is indicated in serious infections caused by susceptible aerobic Gram-positive cocci and anaerobic bacteria for which less toxic alternatives are not indicated. The minimum inhibitory concentration range for Gram-positive cocci is \( \leq 1.0 \) mg/liter (8). The minimum inhibitory concentration range for anaerobic bacteria is 1.6–6.2 mg/liter; however, Clostridia are more resistant than are most anaerobes (9).

Clindamycin and other antibiotics produce colitis in sensitive individuals (10). Present data suggest that the concentrations in blood or stool are not related to this effect.

One case of hepatotoxicity has been reported (11). This was associated with high (25.6 mg/liter) concentrations in serum. Monitoring of serum concentrations in patients with markedly reduced renal or hepatic function may be indicated during high-dose therapy.

Measurement of clindamycin in serum is helpful in diseases such as osteomyelitis (12), when the patient is switched to oral clindamycin after initial intravenous administration. This assay measures only clindamycin and not N-demethylclindamycin, which is also microbiologically active. Therefore, to assess serum antimicrobial concentrations realistically, blood must be drawn between 1 and 2 h after an oral dose, when the parent compound represents 90% or more of the pharmacologically active drug (7). Methylation to convert all of the microbiologically active drug back to the parent drug may provide information on total pharmacologically active drug concentrations at other times. This suggestion (by a reviewer) is being investigated.

We thank the microbiological section of The Upjohn Clinical Research Laboratories, especially W. L. Lummis and M. A. Burnett, for supplying information about the microbiological assay and the results of that assay.

References

3. Morozowich, W., Personal communication.