Bleomycin, an Antitumor Antibiotic: Improved Microbiological Assay and Tissue Distribution Studies in Normal Mice

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A microbiological assay was developed for bleomycin, an antitumor antibiotic reported to be active in human trials. The assay bacterium was a strain of *Escherichia coli* which is resistant to ethionine. Studies revealed relatively high concentrations of bleomycin in the blood and urine of mice after a single dose, < 0.33 LD₁₀, injected intraperitoneally.

*Streptomyces verticillus* produces a group of antibiotics originally designated as bleomycin but subsequently subdivided into bleomycin A and B (13). In turn, bleomycin A was found to consist of six structurally closely related antibiotics, and bleomycin B was found to consist of five structurally closely related antibiotics (14). All of the bleomycins showed marked inhibitory activity against a broad spectrum of gram-positive and gram-negative bacteria. Inhibitory activity against *Ehrlich* carcinoma, sarcoma 180, and other transplantable mouse ascites tumors has also been reported (5, 6, 11, 12). Recent reports indicate that bleomycin is effective against certain types of cancer in humans (2, 3, 7, 10, 15). Microbiological assays for bleomycin employing *Mycobacterium phlei* NIHJ (13) or *Bacillus subtilis* PCT19 (6) have reportedly been used in purification and pharmacological studies, but detailed descriptions of the procedures were not published. Ohnuma et al. (8) recently described in detail a bioassay for bleomycin with *B. subtilis* ATCC6633; the minimal inhibitory concentration of bleomycin was between 0.5 and 2.0 μg/ml, varying with different lots.

The purpose of this communication is to describe a new microbiological assay for bleomycin. This assay is capable of detecting concentrations of bleomycin as low as 0.1 μg/ml in blood. The distribution of bleomycin in blood and tissues of mice is described.

MATERIALS AND METHODS

Previously described methods (4) were used to find an appropriate microorganism for the assay of bleomycin. From approximately 100 species of bacteria tested for sensitivity to this compound, a strain of *E. coli* ATCC9637 resistant to ethionine (designated *E. coli/ETH*) was selected as the assay organism because of its unique sensitivity. The culture was maintained on agar slants of a glucose-salts medium (9) supplemented with 1.0 mg of ethionine per ml. Stationary cultures of *E. coli/ETH* were grown for 16 to 18 hr at 37 C in glucose-salts broth, supplemented with 100 μg of ETH/ml, for the preparation of seeded agar assay plates (100 by 15 mm, Lab-Tex Products, Division Miles Laboratories, Inc., Westmont, Ill.). Cells from these cultures were collected and washed twice by centrifugation in saline (0.85% NaCl), resuspended in saline, and adjusted to 20% light transmittance (660 nm) in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). This suspension was diluted 1:200 with saline, and 10 ml of this suspension was added to 1 liter of cooled (50°C) glucose-salts agar medium supplemented with 10 μg of guanine per ml. Earlier studies with a variety of individual metabolites revealed that the addition of guanine to the medium increased the sensitivity of *E. coli/ETH* to inhibition by bleomycin. A 6-ml amount of the inoculated agar was dispensed into the previously described petri plates.

A stock solution of bleomycin was prepared in sterile saline and was suitably diluted. When 0.08 ml of the various dilutions was added to filter paper discs (1.27 cm in diameter), the following concentrations were obtained (micrograms of bleomycin per disc): 0.008, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0. An additional bleomycin stock solution was prepared in which the drug was dissolved in freshly drawn mouse blood and diluted with saline. Filter paper discs (1.27 cm in diameter, no. 740-E; Schleicher and Schuell Co., Keene, N.H.) were impregnated with 0.08 ml of the respective solutions containing graded concentrations of the drug. The moist discs were placed on the surface of each seeded agar plate and pressed down securely with flamed forceps. All samples and standards were tested in triplicate. Each individual plate contained a maximum of three discs. Two of these discs contained either...
Fig. 1. Standard curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of bleomycin in the body fluids of mice. Assay microorganism: Escherichia coli ATCC9637/ethionine. (A) Blood standard curve (index of precision, 0.049); (B) saline standard curve (index of precision, 0.049).

experimental samples (blood or tissue homogenates) or standard-curve solutions of different concentrations. The third disc contained an empirically selected concentration of 0.1 µg of bleomycin per disc, which allowed for correction of plate-to-plate variation in zone sizes. The plates were preincubated for 24 hr at 4°C and then incubated for 15 to 18 hr at 30°C. The preincubation storage resulted in increased sensitivity. The resulting zones of inhibition on the plates were measured and corrected as previously reported (4). The corrected mean diameters of the zones of inhibition surrounding the discs, which contained known concentrations of drug, were plotted on semilogarithmic graph paper, with the zones sizes on the arithmetic scale and drug concentrations per disc on the logarithmic scale. Standard curves were constructed through the points thus obtained by the method of least squares (1). Drug concentrations in the experimental samples were obtained by reading known drug concentrations on the ordinate of the blood standard curve which corresponded to the size of the corrected zones of inhibition surrounding the discs impregnated with the tissue homogenates.

In all experiments, BDF1 mice (mixed sexes, 18 to 22 g) were used.

RESULTS AND DISCUSSION

E. coli ATCC9637/ETH was selected as the microorganism for the assay of bleomycin because of its sensitivity to this inhibitor, its linear dosage response, and its rapid growth rate. Representative standard assay curves, obtained with bleomycin dissolved in saline and in heparinized freshly drawn whole mouse blood, are shown in

Fig. 2. Concentrations of bleomycin detected in the body fluids of mice by microbiological assay. Each point represents the mean drug level of five mice. Assay organism: Escherichia coli ATCC9637/ethionine. Animals were injected with a single intraperitoneal dose of 20 mg of drug per kg.
Fig. 1. In either diluent, concentrations as low as 0.1 μg/ml could be detected. The concentrations of bleomycin detected in the blood and urine of mice, which were injected intraperitoneally with a 20 mg/kg (<0.33 LD₅₀) dose of drug, are shown in Fig. 2. No bleomycin was detected in the kidneys, lung, spleen, liver, or brain of the mice under these conditions of drug administration, indicating that <0.1 μg of bleomycin per g of tissue was present.

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LITERATURE CITED