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Sensitive Bioassay for Vancomycin

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An accurate and sensitive assay for vancomycin in serum and body fluids has not been available. This paper reports an assay for vancomycin that can detect serum and fluid levels as low as 0.8 μg/ml. A disk diffusion technique was designed employing buffered glucose minimal salts agar and *Bacillus subtilis* as an indicator strain. A linear relationship was obtained between zone diameter and concentration for vancomycin standards from 0.8 to 50 μg/ml prepared in pooled human serum. Results were accurate (<10% error) and reproducible (within-sample standard deviation, 0.25 μg/ml) for concentrations of from 0.8 to 25 μg of vancomycin per ml. Zone diameters were at least 6 mm larger on minimal salts agar than on standard assay media. The increased sensitivity and accuracy of the assay make it possible to accurately measure levels in cerebrospinal fluid and dialyzate fluid as well as in serum.

Vancomycin, a potentially nephrotoxic and ototoxic antibiotic (4), is often the preferred drug of choice in life-threatening infection due to methicillin-resistant *Staphylococcus aureus*. In addition, it has proven useful in preventing shunt infections (3) and in treating severe infection in patients in whom allergy precludes the use of penicillin (4).

As with any potentially toxic antibiotic, it is frequently desirable to monitor patients receiving vancomycin for both therapeutic and toxic levels. This is particularly true in the patient with renal insufficiency, since vancomycin is excreted primarily by the kidneys (2). Serum concentrations in the range of 80 to 100 μg/ml have been associated with serious toxic effects (1). Therapeutic levels are dependent upon the infecting pathogen, but minimal inhibitory and bactericidal concentrations of susceptible strains are usually less than 5 μg/ml. Whereas current bioassay techniques are sufficiently sensitive to determine toxic concentrations, levels of less than 10 μg/ml cannot be measured accurately. Thus, the purpose of this study was to design and evaluate an assay with increased sensitivity that would allow patient serum and fluid levels to be monitored more precisely.

MATERIALS AND METHODS

Assay medium. A minimal salts agar (MSA) was employed in the bioassay. The minimal salts buffer had the following composition: 0.32% KH_2PO_4, 0.42% K_2HPO_4, 0.2% NaCl, and 0.2% $(NH_4)_2SO_4$. Final pH was adjusted to 7.3, and the buffer solution was stable at room temperature for 6 months.

Before a bioassay was performed, 1.5% agar (Difco Laboratories, Detroit, Mich.) was added to the minimal salts buffer, and the solution was resterilized. Filter-sterilized glucose (10% [wt/vol]) was added aseptically to the melted agar medium to give a final concentration of 0.5%. After the medium had cooled to 55°C, 0.1 ml of a sterile mineral salts solution was added for each 100 ml of medium. The mineral salts solution had the following composition: 5.0% $MgSO_4 \cdot 7H_2O$, 0.1% $MnSO_4$, 1.0% $FeCl_3$, and 0.5% $CaCl_3$. The completed MSA was mixed thoroughly, dispensed in 30-ml volumes into petri dishes (100 by 150 mm), and allowed to solidify. Depth of the agar medium was 2.0 ± 0.2 mm. The completed MSA was stable for 1 month when stored in a plastic sleeve at 4°C.

Microorganism. Both a laboratory strain of *Bacillus subtilis* (W23) and *B. subtilis* ATCC 6633 were evaluated as indicator organisms. Since no difference existed between the two strains, all assays were performed with *B. subtilis* W23. Stock cultures were maintained on nutrient agar (Difco) slants at 4°C.

Preparation of standard curve. Stock solutions of vancomycin (Eli Lilly and Co., Indianapolis, Ind.) were prepared by dissolving the antibiotic in sterile distilled water to a concentration of 250 μg/ml. This solution remained stable for up to 10 days if stored at 4°C. To perform the standard curve, a 1:5 dilution of the vancomycin stock solution was made into antibiotic-free heat-inactivated pooled human serum, and serial twofold dilutions were made into sera to give final concentrations of 50, 25, 12.5, 6.3, 3.1, 1.6, and 0.8 μg/ml.

B. subtilis was inoculated into Mueller-Hinton broth (BBL) and incubated at 35°C for 4 to 5 h. The logarithmic phase cells were adjusted to one-half the density of a no. 1 McFarland standard (approximately 5.0×10^7 colony-forming units per ml) in Mueller-Hinton broth. Bioassay plates were inoculated with a sterile cotton swab so as to yield an even lawn of bacterial growth.

Sterile 6-mm blank sensitivity disks (BBL) were saturated by placing them in the standards and the unknown serum or fluid to be assayed. Duplicate disks were placed on opposite sides of the plate. No more than 10 disks were placed on a plate, and high and low concentrations were alternated. The plates were incubated at 35 to 37°C until zone diameters were visible (10 to 12 h). Standard curves were constructed by plotting average zone diameters (in millimeters) against the concentration of the standards (in μg/ml) on four-cycle, 70-division semilog graph paper and joining the points with the best-fit straight line.

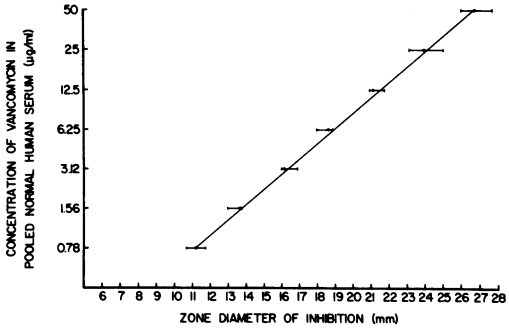

FIG. 1. *Bioassay for vancomycin. This figure represents a composite standard curve, with ranges, derived from mean values of 15 separate standard curves.*

Reproducibility. Assay precision was determined by performing two separate duplicate assays on the same sample on different plates with different standards. Results of each assay were plotted versus the duplicate and observed for adherence to a line of identity.

Comparison of assay sensitivity with other procedures. Standard curves were prepared as described above using Mueller-Hinton agar and Antibiotic Medium no. 5 (Difco Laboratories). Medium depths of both 4.0 mm and 2.0 mm were evaluated, and zone diameters for a given vancomycin concentration were compared with those obtained on MSA.

RESULTS

A composite standard curve representing 15 separate assays is illustrated in Fig. 1. The curve was linear over the range for vancomycin serum levels anticipated after recommended dosages (1 to 20 μg/ml). The lower limit of sensitivity for the assay was 0.8 μg/ml. The ranges of the zone diameters at each standard concentration were never greater than 2 standard deviations from the mean. The greatest variability in zone diameters was found at concentrations of antibiotic >25 μg/ml.

The assay was not affected by varying incubation temperatures between 35 and 37°C. Although zones could be read as early as 10 to 12 h, continued incubation for 16 to 18 h did not

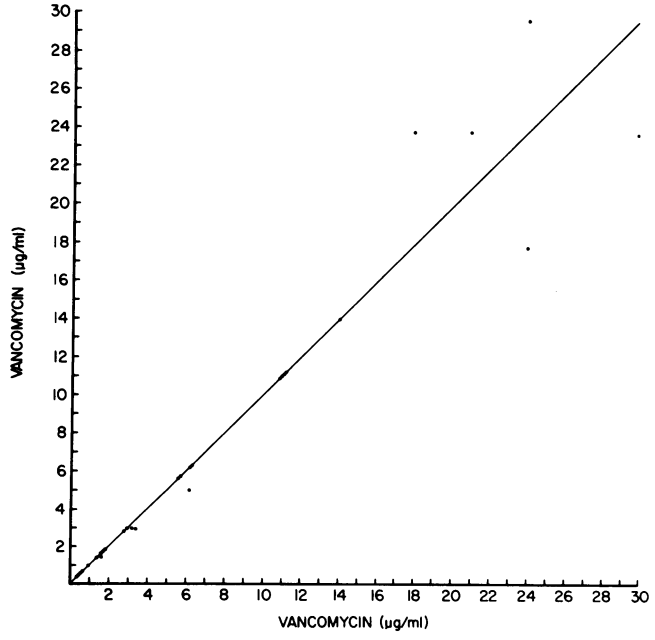

FIG. 2. *Reproducibility of assay results for vancomycin. Each point represents the results of two separate duplicate assays performed on one serum sample, one value plotted on the abscissa and the other plotted on the ordinate. The line of identity is included for reference.*

alter zone diameters, nor did it cause the zones to become fuzzy.

Accuracy of the technique was evaluated by testing sera with known vancomycin concentrations. Results were within 10% of the known value for concentrations ≤25 μg/ml.

The precision of the assay technique is illustrated in Fig. 2. The closeness of the paired values to the line of identity confirms the degree of reproducibility of the technique. The within-sample standard deviation (SD) was calculated according to the equation $SD = (\Sigma d^2/2n)^{1/2}$, where d is the difference in micrograms per milliliter between the results of the two assays of the same specimen, and n is the number of specimens. The within-sample SD of each pair of results was 1.7 μg/ml for concentrations ranging from 0.78 to 50 μg/ml. If concentrations >25 μg/ml were excluded, the within-sample SD was 0.25 μg/ml. This emphasizes the loss of reproducibility at higher antibiotic concentrations with this assay technique.

A comparison of the sensitivity of the MSA medium to Mueller-Hinton agar and Antibiotic Medium no. 5 is illustrated in Fig. 3. Zone diameters obtained on MSA for the concentrations of vancomycin assayed were at least 6.0 mm larger than comparable concentrations tested on Mueller-Hinton agar or Antibiotic Medium no. 5. This was true even if agar depth was reduced to 2.0 mm with the standard assay media.

This technique has been used to monitor several patients receiving vancomycin therapy. The hospital course of a representative patient appears in Fig. 4. The patient, a 22-year-old white female, was admitted to St. Joseph's Hospital, Omaha, Neb., with a diagnosis of staphylococcal endocarditis and acute renal failure secondary to methicillin-induced nephritis. After the development of a hypersensitivity reaction to cephalothin, the patient was

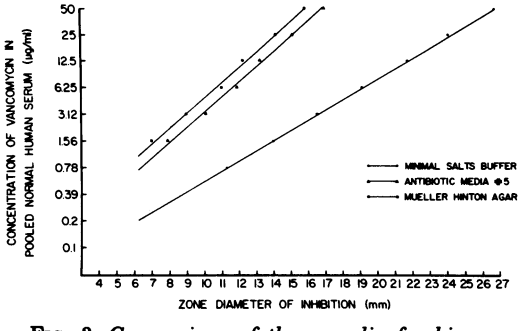

FIG. 3. *Comparison of three media for bioassay of vancomycin. Each curve was performed using a medium depth of 2.0 mm.*

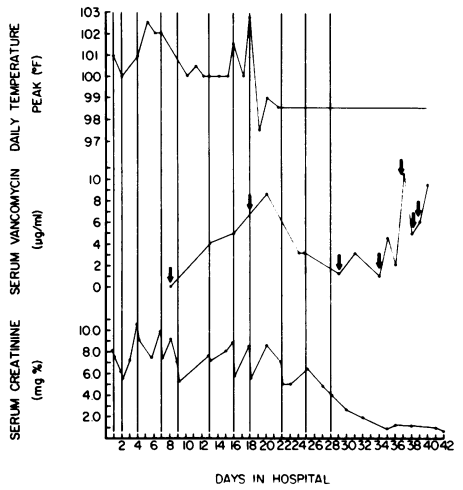

FIG. 4. *Patient's hospital course as influenced by vancomycin. Vertical lines represent hemodialysis; arrows represent 1-g dose of vancomycin administered intravenously.*

placed on vancomycin. Vancomycin assay allowed use of an interrupted dosage schedule, and serum levels were maintained below 12 μg/ml. The patient remained afebrile except for two isolated fever spikes on days 8 and 10 of vancomycin therapy, and renal function improved over the hospital course. After six consecutive negative blood cultures, the patient was discharged and remains well 13 months after therapy.

DISCUSSION

The standard agar diffusion assays for vancomycin are not sufficiently sensitive to detect levels below 10 to 12 μg/ml. The technique reported here allows accurate determination of vancomycin levels between 0.5 and 25 μg/ml. Concentrations above 50 μg/ml can also be determined, although the assay becomes less accurate and less reproducible at higher concentrations. It is therefore recommended that 1:5 and 1:10 dilutions of unknown sera be tested as well as the undiluted specimen to insure obtaining at least one accurate result.

The assay employs reagents and an organism that are readily available to the clinical laboratory. In addition, the minimal salts buffer and the completed MSA plates are stable for a relatively long period of time. Although zone diameters could be read as early as 10 to 12 h, continuing incubation for 16 to 18 h did not alter results, nor did incubation at 37°C rather than at 35°C.

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