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Microassay for Amphotericin B

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Depending on the hematocrit, duplicate or triplicate determinations of serum amphotericin B concentration may be made on as little as 100 μl of capillary blood obtained by finger prick. In an accurate plate diffusion bioassay, using *Paecilomyces varioti* as the indicator organism, levels of the drug in the therapeutic range can be determined fast enough for clinicians to modify their next dose.

Traditional bioassay methods for amphotericin B (1, 2) require quantities of blood which are most conveniently obtained by venipuncture. Since therapy with amphotericin B is often prolonged, repeated venipuncture over a long period for the purpose of following serum levels of the drug is distressing to the patient and inconvenient for the clinician. Also, in the pediatric age group, particularly in neonates, it is often impractical to obtain blood regularly in this way. This report describes a miniaturized amphotericin B bioassay system utilizing only 100 μl of capillary blood collected from the heel or by finger prick and suitable for determining the amount of this drug in serum.

MATERIALS AND METHODS

The assay method of Shadomy et al. (2), with minor modifications, was used.

Media and cultures. *Paecilomyces varioti* (MSSC 5605, National Institute of Allergy and Infectious Diseases) was grown at 37°C on dry slants of unmodified Sabouraud dextrose agar for 2 to 3 days. Mature cultures of *P. varioti* consisting mostly of conidia were harvested by washing with sterile saline, and the spore suspension was adjusted to a light transmission of 30% at 360 nm on a Bausch & Lomb Spectronic 20 spectrophotometer (approximately 5×10^6 spores/ml). This suspension remains stable for 5 days at 4°C.

Sterile, melted agar (nystatin assay agar, Difco) in 150-ml volumes was cooled to 50 to 52°C and inoculated with the adjusted spore suspension of *P. varioti* at a 1% (vol/vol) ratio. The inoculated medium was then well mixed without excess agitation and dispensed in 15-ml volumes with a wide-bore serological pipette into sterile plastic petri dishes (150 by 15 mm; Fisher or Falcon) placed on a special, heated level surface (Bio-Tech Co., Canada). After the plates had set, they were held at 4°C for at least 1 h. Prior to use the cooled plates were dried at 37°C for 10 min to remove excess surface moisture, and 21 wells were cut with a 4.5-mm cork borer in a formation such as to minimize the variation in zone size as a result of unevenness of the agar depth. Before filling the wells any excess moisture in them was removed with a sterile disposable pipette.

Preparation of stock drug solution for use in the dose response curve. Amphotericin B (E. R. Squibb & Sons, Inc.), 50 mg of active drug, was dissolved in 5 ml of 100% dimethyl sulfoxide. This was diluted 1:10 in sterile 60% dimethyl sulfoxide to give a solution containing 100 μg/ml. This stock solution is stable for at least 4 months if stored at −70°C. Further dilutions were prepared freshly for each experiment by diluting the stock solution 1:20 in alkalinized 50% isopropyl alcohol (6 drops of 10% NaOH plus 100 ml of diluted alcohol) to give a solution containing 50 μg/ml. This solution retains its potency for up to 7 days if stored at 4°C.

Dose response curve. The 50-μg/ml amphotericin B solution was diluted in pooled normal human serum (heat inactivated and filtered) and 5 μg/ml. Using fresh pipettes for each transfer, doubling dilutions in serum were made from 2.5 to 0.078 μg/ml. Heparinized capillary tubes (Instrumentation Laboratory) of 100-μl volume were filled with each of the standards and, using a micro-rubber bulb to expel the fluid, the wells were filled in duplicate. Plates were incubated on a level surface at 30°C for 24 h. Zones of inhibition were measured in two diameters against a 1-mm square grid taped to the stage of a Bausch & Lomb plate microscope. The standard curve was obtained by plotting the average zone diameter (in millimeters) against the concentration of the standards (in micrograms per milliliter) on 4-cycle, −70-division semilog graph paper and joining these with the best-fit straight line.

Preparation of biological fluids for assays. Capillary blood obtained from a heel or by finger prick was collected in 100-μl heparizined tubes. The cells were packed by centrifugation at 1,000 rpm for 1 min in a microhematocrit centrifuge (Clay-Adams Inc., New York) and separated from the serum by filing through the capillary tube at the cell-serum interface. A micro-rubber bulb was used to expel the serum from the sawed capillary tube and to fill duplicate wells with approximately 20 μl. Depending on the hematocrit, two or three determinations could easily be made from the 100-μl heparinized capillary samples. Using a 21-well template, it was possible to assay in duplicate seven standards

and three patient samples on one plate. Plates were incubated and read as above.

Reproducibility. Assay precision was assessed on 27 specimens by performing the determination twice (i.e., two sets of duplicates) on different plates with the same or different standards. "Within sample" standard deviation (SD) of the paired results was calculated according to the equation SD = $(\sum d^2/2n)^{1/2}$, 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.

RESULTS

A composite dose response curve derived from the measurements on 124 separate curves is shown in Fig. 1. The curve was linear over the normal range for amphotericin B serum levels at recommended doses. The lower limit of sensitivity was of the order of 0.07 μg/ml. The indicated ranges of the zones of diameter at each of the standard concentrations were within acceptable limits.

The precision of the assay technique is shown in Fig. 2. The closeness of the paired values to the line of identity emphasizes the high degree of reproducibility. The within sample SD of each pair of results was 0.026 μg/ml.

Figure 3 depicts the correlation between daily incremental doses of amphotericin B in a representative case during week 1 of therapy and the serum levels of the drug obtained by this assay method when specimens were withdrawn at 1 to 3 h after the end of each infusion. The almost linear relationship of dose to level and the capacity of the assay to detect low concentrations of amphotericin B after even small doses provide an indication of the accuracy of this method in a clinical setting.

DISCUSSION

The standard agar diffusion assay of Shadomy et al. (2), on which the present miniaturized version is modeled, requires 0.2 ml of serum for duplicate determinations. In the absence of details on the need for special processing of the blood samples from which these quantities of serum are obtained, it is presumed that collection was by the traditional method of venipuncture. Depending on the hematocrit, the assay described here requires only one-fifth as much serum (approximately 40 μl) conveniently collected as heparinized capillary blood. The nature of the standard curve, the high degree of reproducibility of the technique, and the excellent correlation between dose and serum level illustrated in Fig. 1, 2, and 3 attest to the accuracy and sensitivity of this micromethod for amphotericin B assay. With usual doses of amphotericin B, serum levels rarely exceed 2.01 μg/ml (1) and, although the assay as described covers the expected range of serum levels, additional studies not reported here confirm that much higher levels can be measured with equal accuracy.

The microassay method offers advantages in two principal situations. First, repeated, even

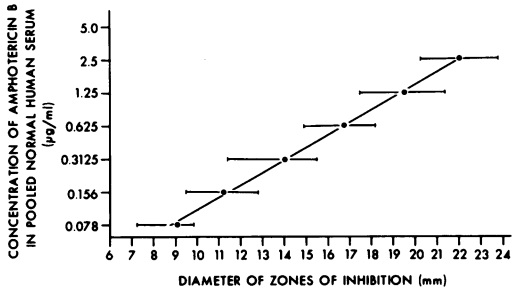

FIG. 1. *Bioassay for amphotericin B. This represents a composite dose response curve, with ranges, derived from mean values for 124 separate dose response curves.*

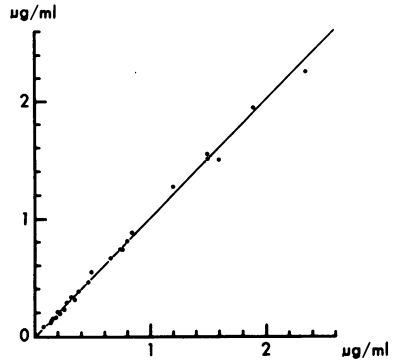

FIG. 2. *Reproducibility of assay results for amphotericin B. Each point represents the results of two separate duplicate assays performed on one serum sample, one value plotted on the ordinate and the other plotted on the abscissa. The line of identity is included for reference.*

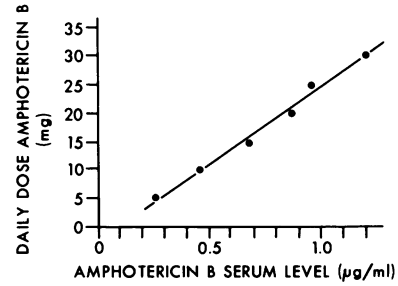

FIG. 3. *Relationship between daily dose of amphotericin B and assayed serum level (patient's weight, 69.3 kg).*

daily, patient sampling throughout the long courses of amphotericin B therapy generally employed now becomes possible. Serum levels can be accurately monitored and correlated with biochemical indexes of altered renal function, and ultimately the determination of a "toxic" amphotericin B level can be achieved. The second major area of attraction is in pediatrics, where economy of blood sampling, particularly in the neonatal age group, is desirable. In addition, from a pharmacokinetic viewpoint a neonate is unique in the way that it handles drugs and, although little is known of the fate of amphotericin B in an adult, its behavior in a neonate is a totally uncharted region. With the provision of a microtechnique for assaying amphotericin B in the above circumstances, perhaps the answers to many of these questions will be forthcoming.

ACKNOWLEDGMENTS

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