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Amphotericin B Serum Concentrations During Therapy[1]

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Therapeutic outcome of patients being treated for systemic mycoses with amphotericin B is possibly related to the serum concentrations of this drug that are produced in these patients. Because current data are conflicting, the magnitude of these concentrations was restudied by using a bioassay which gave precise and accurate results. The highest of 155 serum concentrations was 2.01 μg/ml. Mean concentrations were 1.21, 0.62, and 0.32 μg/ml, at 1, 18, and 42 hr, respectively, after intravenous infusion of amphotericin B. This drug was detected in serum 7 weeks after completion of treatment, but it could not be detected 13 weeks after treatment. Drug levels did not appreciably decrease in serum stored for 8 to 9 months at −10 C. Unequal serum content in assay tubes and measurement of assay turbidity by visual inspection may explain previously reported amphotericin B levels of 3.0 to 12.5 μg/ml.

Recent data on treatment of systemic mycoses with amphotericin B support the possible relation between serum concentrations of this agent and therapeutic outcome (7, 8). These reports suggest that therapy would be more effective if serum concentrations were repeatedly made to exceed by some factor the minimal inhibitory concentration (MIC) of amphotericin B for the infecting fungal strain. A similar concept is widely accepted in the treatment of systemic bacterial infections (4, 9, 20, 28).

The reported serum concentrations of amphotericin B in patients vary significantly. Some investigators (1, 3, 8, 16, 18, 19, 23, 27, 29) report levels of 1.0 to 2.4 μg/ml, whereas others (7, 31) report levels of frequently 3.0 and occasionally 6.0 to 12.5 μg/ml. MIC values of amphotericin B for common pathogenic fungi often approach or exceed 1.0 μg/ml (7, 16, 19, 23). Thus, it is important from both clinical and investigative standpoints to determine which of the conflicting observations correctly approximates serum concentrations of amphotericin B obtainable in patients.

To clarify this question, serum concentrations of amphotericin B were measured in patients receiving treatment for deep mycoses. Precision and accuracy of the serum assay for this agent have been studied, as has the stability of amphotericin B in serum stored at −10 C. Results indicate that discrepancies in magnitude of serum concentrations previously reported reflect inadequate assay techniques.

MATERIALS AND METHODS

Assay for amphotericin B in blood serum. The method designed by Platt (*personal communication*) was modified slightly. Growth inhibition of a strain of *Candida tropicalis* produced by dilutions of a patient's serum was measured photometrically and compared in parallel with inhibition produced by serum containing known concentrations of amphotericin B. Results were expressed in micrograms of amphotericin B anticandidal activity per milliliter of serum.

In preparing the assay inoculum, cultures of *C. tropicalis* (Squibb strain 1647) were maintained by weekly transfers on Sabouraud agar. Inoculum medium contained the following: glucose, 11 g; yeast extract, 6.5 g; tryptone (Difco), 10.0 g; beef extract, 1.5 g; peptone, 5.0 g; distilled water, 1 liter. This medium was sterilized at 121 C for 20 min, and 10^5 units of penicillin and 125 mg of streptomycin were added. Assay medium contained the following: Casitone (Difco), 9.0 g; glucose, 20.0 g; yeast extract, 5.0 g; sodium citrate·$2H_2O$, 10.61 g; $NaH_2PO_4 \cdot H_2O$, 1.0 g; $Na_2HPO_4 \cdot 7H_2O$, 1.0 g; distilled water, 1 liter; *p*H 6.8. This medium was sterilized for 20 min at 121 C, and 10^6 units of penicillin and 1 g of streptomycin were added. A slant growth of *C. tropicalis* was suspended in 10 ml of inoculum medium, and one drop of this suspension was added to 100 ml of inoculum medium.

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This was incubated for 12 to 14 hr at 37 C on a rotating shaker at 100 rev/min until a transmittance of 65%, as measured with a Bausch & Lomb Spectronic-20 colorimeter (550 nm), was achieved. A 0.02-ml amount of the resulting culture was added to 100 ml of assay medium.

Samples of serum containing known concentrations of amphotericin B (standards) were prepared with an assay standard obtained from the Squibb Institute for Medical Research. After desiccation under vacuum for 1 hr, 10 mg of active standard was dissolved in 100 ml of dimethyl sulfoxide (DMSO) and then diluted 1:10 with 60% DMSO. This solution was diluted into pooled human serum so that standards contained the following concentrations of amphotericin B: 0.0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, and 1.0 μg/ml. The final concentration of DMSO in the assay did not inhibit growth of the test organism.

Serum samples to be assayed were diluted with pooled human serum to give final dilutions of undiluted, 1:2, 1:3, 1:4, 1:6, 1:10, 1:12, 1:16, 1:20, and 1:30.

A 0.2-ml amount of a standard was pipetted into each of three test tubes (13 by 100 mm), and 0.2 ml of a dilution of sample was pipetted into each of four test tubes. Then 0.2 ml of inoculum was dispensed into all tubes containing standards and into three of the four tubes containing each dilution of sample. Sterile assay medium in 0.2-ml portions was then dispensed into tubes containing uninoculated dilutions of samples. Tubes were incubated for 20 hr at 30 C. Growth was terminated by adding 3 ml of 4% formaldehyde to each tube. Per cent light transmittance (%T) of standards and dilutions of samples was measured in a Bausch & Lomb Spectronic-20 colorimeter at 430 nm with an 11-mm cuvette. Infinite light transmission was adjusted to 80% by using the 1.0 μg/ml standard for the standards and by using the contents of the uninoculated tube for each dilution of the samples.

A dose-response curve was constructed by plotting log concentration (micrograms per milliliter) versus the average of triplicate %T readings of each standard. Analysis of variance (6) demonstrated linearity in six of seven randomly selected curves. The anticandidal activity (micrograms per milliliter) of a sample dilution, with %T value on the linear part of this curve, was read from the log scale. This result was multiplied by the reciprocal of the dilution to give total anticandidal activity of the patient's sample. Serum concentration of amphotericin B was determined by subtracting the anticandidal activity of a pretreatment serum sample from total anticandidal activity of the posttreatment serum sample. Allowance was made for pretreatment activity because of the capacity of human serum to inhibit growth of certain fungi (2, 21, 22). In this study, pretreatment samples were available from only 10 of 20 patients; thus, the average of the 10 pretreatment results, 0.06 μg/ml (range, 0.02 to 0.16 μg/ml), was subtracted from each assay result from the remaining 10 patients.

Assay precision. Assays were performed twice on the same serum specimen and were done on different days within 1 month after venapuncture. Thirty-four assays were performed on samples of 17 specimens. "Within-sample" standard deviation (SD) of the paired results was calculated by using the equation (25) $SD = (\sum d^2/2n)^{1/2}$, where d is the difference in micrograms per milliliter between results of two assays of the same specimen and n is the number of specimens.

Assay accuracy. Samples of serum from seven patients were assayed by both the Squibb Institute for Medical Research and by our laboratory. Results obtained by the two laboratories were compared by the method just described (25).

Stability of amphotericin B in stored serum. Samples of the same specimen stored at −10 C were assayed on two occasions. The initial assay of each specimen was performed within 1 month after venapuncture, and the second assay was performed 8 to 9 months after venapuncture. Forty assays on 20 specimens were performed in this manner.

Patients studied. We obtained 155 serum samples for amphotericin B assay from 20 patients who were receiving treatment for systemic fungal infections. Amphotericin B was initially given in doses which were gradually increased to 50 mg (0.6 to 1.0 mg/kg); this dose was then given intravenously (iv) over 4 to 6 hr. Blood samples were collected aseptically 1, 18, 42, and 66 hr after completion of 50-mg iv infusions; these were taken when possible after the 150-, 500-, 1,000-, and 2,000-mg cumulative doses. Samples were also obtained up to 13 weeks after completion of all treatment in five patients who had received assays of their pretreatment serum. Serum was divided into 2-ml samples and stored at −10 C within 1 hr after collection.

Ten of 49 serum specimens obtained 1 hr after infusion were taken from patients receiving daily 50-mg doses of amphotericin B. Another 10 were obtained when either the serum urea nitrogen (26) was greater than 40 mg/100 ml or the endogenous creatinine clearance (5) was less than 35 cc/(min × 1.73 m² of body surface area). Two additional 1-hr specimens were collected in the presence of both marked renal dysfunction and daily 50-mg doses. For these reasons, we believe that the serum concentrations of amphotericin B reported herein reflect those occurring under most circumstances.

RESULTS

Figure 1 and Table 1 show the serum concentrations of amphotericin B observed in this study. The highest concentration was 2.01 μg/ml. One hour after completion of iv infusion, 86% of the levels ranged between 0.80 and 1.60 μg/ml. At 18 hr, 87% of the values were between 0.30 and 0.90 μg/ml. By 42 hr, the levels had further declined so that 85% were between 0.17 and 0.45 μg/ml. Amphotericin B was detected in the serum of one of two patients studied 7 weeks after completion of treatment; however, no antimicrobial activity was demonstrable in a single patient studied 13 weeks after treatment.

Figure 2 illustrates the disappearance of amphotericin B after iv administration of the drug to

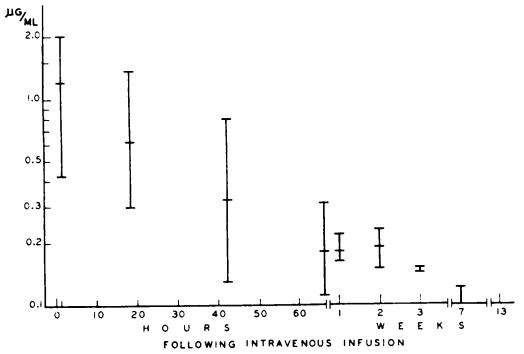

FIG. 1. *Serum concentrations of amphotericin B. Mean and range of concentrations are plotted logarithmically on the ordinate against time after completion of intravenous infusion plotted on the abscissa.*

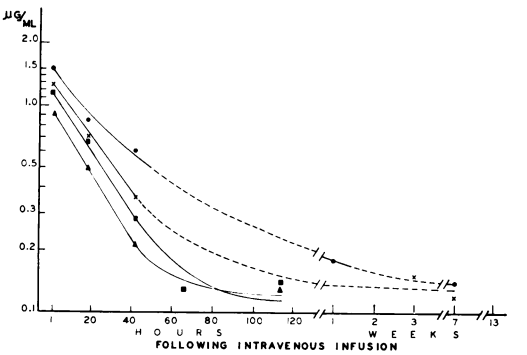

FIG. 2. *Disappearance of amphotericin B from serum after completion of intravenous infusion in four patients. Serum concentrations are plotted logarithmically on the ordinate against time plotted on the abscissa. Curves are drawn by inspection; dashed areas represent extrapolation.*

TABLE 1. *Serum concentrations of amphotericin B*

Time after drug administration[a]	Concn (μg/ml)		No. of concn measured	No. of patients studied
	Mean[b]	Range[b]		
1 Hr	1.21	0.42–2.01	49	18
18 Hr	0.62	0.27–1.38	47	20
42 Hr	0.32	0.13–0.79	34	16
66 Hr	0.18	0.11–0.31	7	7
90 Hr	0.22	0.13–0.35	3	3
114 Hr	0.14	0.13–0.14	3	3
1 Week	0.18	0.16–0.22	5	5
2 Weeks	0.19	0.15–0.23	2	2
3 Weeks	0.14	0.14–0.15	2	2
7 Weeks	0.06	0–0.12	2	2
10 Weeks	0	0	1	1

[a] After completion of intravenous infusion of amphotericin B.

[b] Results are not necessarily sequential concentrations observed in the same patient.

four patients. It is seen that the drug does not disappear in an exponential (linear) fashion; however, not enough data points were obtained to assign a mathematical model to this disappearance pattern or to allow a statement that any subdivided portion of the curve was linear.

The precision of the assay technique is shown in Fig. 3. The proximity of duplicate values to a line of identity emphasizes the high degree of reproducibility. The within-sample SD of each pair of results was 0.09 μg/ml (25).

Table 2 summarizes the accuracy of this technique. There is close agreement in assay results of the same sample obtained by the two laboratories. The within-sample SD of each pair of values was 0.13 μg/ml (25).

The stability of amphotericin B in serum stored for 8 to 9 months at −10 C is shown in Fig. 4.

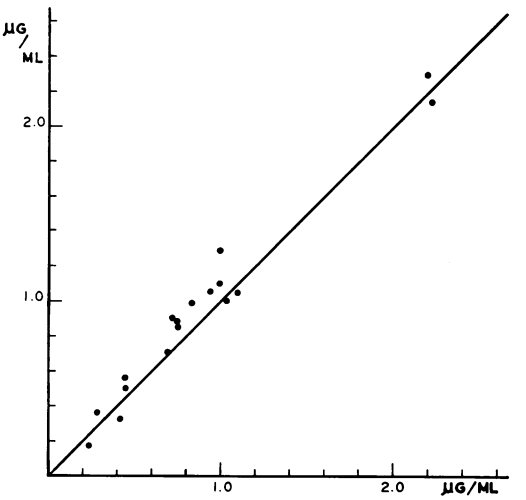

FIG. 3. *Reproducibility of assay results for amphotericin B. Each point represents the results of two separate assays performed on one serum sample, one value plotted on the ordinate and the other plotted on the abscissa. A line of identity has been drawn for clarity. (The four highest results were observed under unusual conditions and are not reported in the text.)*

There was no apparent change in concentration of this antimicrobial during this interval.

DISCUSSION

The serum concentrations of amphotericin B observed in this study are consistent with those reported in other recent studies using reproducible assay techniques. Battock et al. (1) reported concentrations of 0.02 to 1.4 μg/ml as determined by Platt's method, and Bindschadler

TABLE 2. *Concentrations of amphotericin B assayed in different laboratories*

Serum specimen no.	Concn (μg/ml)	
	Squibb Institute laboratory	Our laboratory
240	1.10	1.10
245	1.10	1.07
246	0.66	0.46
249	1.40	1.84
256	1.40	1.35
263	0.95	0.93
U-9	1.00	1.00

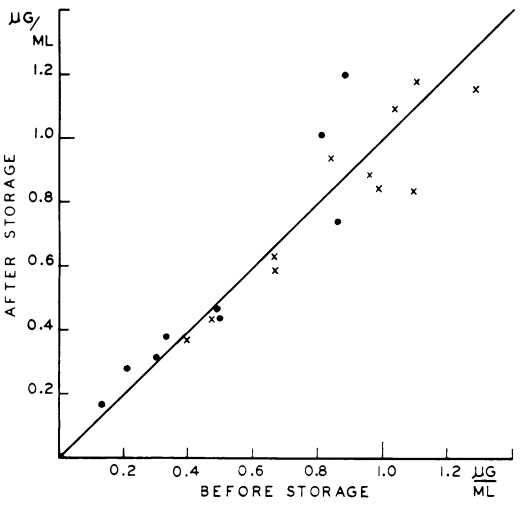

FIG. 4. *Stability of amphotericin B in serum samples stored at −10 C for 8 (●) to 9 (×) months. Each point represents two separate assays performed on one serum sample, the result of the assay performed before storage being plotted on the abscissa and the assay value obtained after storage plotted on the ordinate. A line of identity has been drawn for clarity.*

and Bennett (3) observed mean serum concentrations of 0.1 to 2.4 μg/ml with an agar-diffusion technique.

Review of the tube-dilution methods of assay of amphotericin B which have been used in the past reveals several factors which could explain the discrepancies in magnitude of reported serum concentrations of this agent. First, the requirement for using pooled human serum rather than broth medium as the diluent for the serum to be assayed could be involved. Gerke et al. (10) have determined that the content of human serum must be equal in every tube of the assay for amphotericin B because serum influences the activity of this antimicrobial. The effect of serum on amphotericin B activity was reported by Louria (18), shortly after Gottlieb et al. (11) showed that sterol compounds protected strains of fungi from the antimicrobial activity of amphotericin B and other polyenes. Lampen et al. (15) demonstrated that cholesterol reversibly reduced the ultraviolet absorbance of colloidal suspensions of polyene antimicrobials. This indicated physicochemical interaction between cholesterol and polyenes resulting in lower effective concentrations of the polyenes. Rebell (21) demonstrated that Cohn Fraction III-O of human plasma protein, human albumin, and a nonlipoprotein fraction of plasma reduced the inhibitory effect of amphotericin B on a strain of *C. albicans*, whereas cholesterol alone had no effect on the antifungal activity of this drug. Certain concentrations of whole plasma also reduced the anticandidal properties of this agent.

These data strongly suggest that part of the antimicrobial property of amphotericin B is bound stoichiometrically in serum, probably to lipoproteins. Thus, the use of broth medium to serially dilute the serum sample to be assayed may also progressively dilute a factor inhibiting amphotericin B and may lead to a falsely elevated result. Kramer et al. (14), Shadomy et al. (24), and those previously mentioned (1, 3, 10) have already used pooled human serum as a diluting fluid in amphotericin B assays. Other investigators (12, 13, 17) have recommended using serum or protein substitutes in assays of nonpolyene antimicrobials which are partly bound to serum proteins.

Second, determining end point of turbidity by visual inspection in tube-dilution assays could partly explain the discrepancies in reported serum levels of amphotericin B. Visual inspection is simpler than photometric turbidimetry; however, the coefficient of variation of this step can reach 100% (10) when twofold dilutions are used.

Finally, variable errors in assay of amphotericin B will occur if allowance is not made for the fungistatic effect of human serum (2, 21, 22).

These sources of error in assay for amphotericin B generally appeared in the methods used in studies reporting serum levels of 3.0 to 12.5 μg/ml. It is therefore postulated that these levels of amphotericin B may have been lower than indicated by the assay results. Shadomy et al. (24) recently reported serum concentrations of 4.6 to 9.0 μg/ml, but these occurred after extraordinarily high doses of amphotericin B.

Bindschadler et al. (3), Louria (18), and Vogel et al. (30) noted persistence of amphotericin B in serum for extended periods after completion of treatment, and the data on decay of serum levels

of this agent reported herein are consistent with their findings. These observations emphasize the need for more information on the tissue distribution of amphotericin B.

The use of antimicrobial assays and fungal susceptibility tests to study the treatment of systemic mycotic diseases may offer promise. If an optimum ratio of serum concentration to MIC can be determined for a given disease, it might then be possible to determine the dosage which would be adequate for most patients. Using this information, mortality and disability might be kept to a minimum even without the expensive laboratory procedures described herein. By repeatedly producing an optimum ratio of serum concentration to MIC while varying duration of treatment in different groups of patients, it might also be possible to determine how long antimicrobial therapy should be continued. A prerequisite for accomplishing these goals is the use of assay procedures which give reasonably accurate results. Clearly it will be misleading and possibly hazardous to depend upon a ratio of serum concentration to MIC of two, if the technique for measuring these concentrations includes a coefficient of variation of as high as 100%.

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