Rapid Determination of Amphotericin B Levels in Serum by High-Performance Liquid Chromatography without Interference by Bilirubin

HIDEO HOSOTSUBO,¹* JUN TAKEZAWA,² NOBUYUKI TAENAKA,² KIKUMI HOSOTSUBO,² AND IKUTO YOSHIYA²

Central Laboratory for Clinical Investigation¹ and Intensive Care Unit,² Osaka University Hospital, 1-1-50, Fukushima-ku, Osaka 553, Japan

Received 29 December 1987/Accepted 21 April 1988

Amphotericin B (AMB) is a polyene antifungal agent which was isolated from Streptomyces nodosus in 1956 (10) and remains today the drug of choice for the treatment of systemic fungal infections (5, 12). The most frequent and clinically significant side effect of AMB therapy is nephrotoxicity (8). Although toxicity is generally dose dependent, there is considerable interindividual variability among patients. Because of these large individual variations in AMB levels in serum with oral administration, a method is required for frequent and rapid estimation of drug levels which provide adequate antifungal activity but do not produce adverse effects.

The concentrations of AMB in serum were previously determined by microbiological assays (2, 11). Although bioassays have been used traditionally for the determination of concentrations of antibiotics, they have limited sensitivity and reproducibility. The additional disadvantages of bioassays are their lack of specificity and the length of time required for sample analysis. High-performance liquid chromatography (HPLC) provides a sensitive and specific alternative to bioassays, with the additional advantages of high precision and rapid turnaround time. We developed a sensitive HPLC assay for the measurement of AMB in serum and compared it with the microbiological assay.

The HPLC assay was performed with an LC-6A pump, an SPD-6AV UV-visible variable-wavelength detector, and a C-R4A computing integrator (Shimadzu, Kyoto, Japan). Analysis was performed on a reversed-phase CLC-trimethylsilyl column (5 μm; 150- by 6.0-mm [inside diameter]; Shimadzu). Samples were injected with a Rheodyne syringe-loading sample injector (model 7125; Shimadzu) fitted with a 50-μl loop. The mobile phase for HPLC analysis consisted of an acetonitrile–10 mM acetic buffer (pH 7.4) mixture (40/60, vol/vol). The mobile phase was degassed and delivered with a flow of 1.0 ml/min at room temperature. The A₄₅₀ of the column effluent was monitored (0.005 absorbance units full scale).

Serum samples used in this study were obtained from patients with systemic fungal infections receiving AMB as well as other antibiotics for which various determinations of concentrations in serum had been ordered as part of the clinical management of the patients. Serum standards and controls were prepared by dissolving AMB (Japan Squibb, Tokyo, Japan) in dimethyl sulfoxide and diluting the solution in drug-free pooled human serum samples with concentrations of 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 μg/ml for standards and 0.1, 0.3, and 0.7 μg/ml for controls. Standard and control sera were dispensed as 1.0-ml samples and stored at −70°C.

Sample preparation for the HPLC assay required only protein precipitation. A 0.2-ml volume of serum sample or standard or control serum was mixed vigorously for 10 s with 0.2 ml of acetonitrile. The mixture was centrifuged at 12,000 × g for 2 min. Following centrifugation, 50 μl of the supernatant was injected into the HPLC system.

HPLC determinations of concentrations in serum were compared with those from a microbiological assay. AMB concentrations in serum were assayed by a modification of the method of Shadomy et al. (11) by using Paecilomyces variotii as the test organism.

Typical chromatograms for extracts of blank serum, serum spiked with AMB, and patient serum with a high concentration of bilirubin are shown in Fig. 1. The peak of interest was eluted in less than 10 min. More than 37 commonly used drugs were tested and shown not to interfere with this assay. These included 14 antibiotics (amikacin, ampicillin, benzylpenicillin, carbenicillin, cefoxitin, cefuroxime, chloramphenicol, flucytosine, gentamicin, kanamycin, ketoconazole, miconazole, penicillin G, and tobramycin), 9 antineoplastics (adriamycin, allopurinol, cisplatin, cyclophosphamide, cytarabine, dactinomycin, 5-fluouracil, methotrexate, and thioguanine), acamitomophen, acetazolamide, aspirin, carbamazepine, chlorpromazine, diazepam, ethosuximide, furosemide, phenobarbital, phenytoin, primidone, procainamide, quinidine, and theophylline.

Analytical recoveries of added AMB from serum by this extraction procedure (mean ± standard deviation; n = 6) were 102 ± 3, 97 ± 2, and 102 ± 2% at concentrations of 0.08, 0.24, and 0.48 μg/ml, respectively. The standard curve was generated by least-squares linear regression analysis of the peak height of AMB. The standard curve was linear over the range of drug concentrations used (0.05 to 2.0 μg/ml; n =

* Corresponding author.
and accurate measurement of AMB concentrations in serum without potential interference by bilirubin. This method has the rapidity (10 min per injection), specificity, and simplicity required for routine use in the clinical laboratory.

FIG. 1. Chromatograms of (A) drug-free blank human serum, (B) drug-free serum spiked with 0.5 \( \mu \)g of AMB per ml, and (C) serum collected from a patient with a systemic fungal infection and hyperbilirubinemia after intravenous administration of 24.0 mg of AMB per day. (The calculated concentration of AMB was 0.7 \( \mu \)g/ml, and the bilirubin concentration was 23.3 mg/dl.)

6) and passed through the origin, with a correlation coefficient of 0.999. To define within- and between-day precision for serum specimens with the HPLC method, we analyzed each of the three control samples (0.1, 0.3, and 0.7 \( \mu \)g/ml) 10 times in a single run and 6 times in separate runs. The coefficients of variation ranged from 1.02 to 2.11% for within-day precision and 2.88 to 4.32% for between-day precision.

In a direct comparative study, 50 serum specimens from patients receiving AMB were assayed by both the HPLC method and microbiological assay. The concentrations determined by the two methods were compared with each other by linear regression (Fig. 2) and showed good agreement. The correlation coefficient was 0.984, and the equation of the regression line was \( y = 0.93x - 25.7 \).

Use of a bioassay to measure AMB in serum is complicated by a number of factors, including long incubation times, poor reproducibility, and interference from concomitantly administered antifungal agents. Although several other HPLC methods for AMB have been described elsewhere (1, 3, 4, 6, 7, 9, 12), most have required many steps in sample preparation (1) or have a very large coefficient of variation (7). The HPLC method of Granich et al. (4) for AMB determination required a solid-phase extraction procedure for elimination of potential bilirubin interference. Using an analytical column (CLC-trimethylsilyl; 5 \( \mu \)m), we effectively eliminated bilirubin from the sample. Another advantage of the current assay is the simplicity of the extraction procedure. The present method involves only protein precipitation with acetonitrile. Analytical recovery of AMB is approximately 100%.

In summary, the small sample size (0.2 ml) required for this method is an advantage for therapeutic monitoring of AMB in patients. In addition, this method permits precise
LITERATURE CITED