Sensitive High-Pressure Liquid Chromatographic Assay for Amphotericin B Which Incorporates an Internal Standard

GEORGE G. GRANICH, GEORGE S. KOBAYASHI, AND DONALD J. KROGSTAD

Microbiology and Therapeutic Drug Monitoring Laboratories, Barnes Hospital, and the Departments of Pathology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

Received 7 October 1985/Accepted 9 January 1986

Amphotericin B is a polyene antifungal agent that is presently the drug of choice for the treatment of most severe systemic fungal infections. Use of the drug, however, is limited by its toxicity, particularly by its impairment of renal function. The pharmacokinetics of amphotericin B are complex, involving a rapid initial decrease in serum levels for approximately 24 h, followed by a long (15-day half-life) terminal elimination phase (1, 4, 8). Other pharmacokinetic properties, such as elimination pathways, are not completely understood. Renal and biliary excretion have each been estimated to account for up to 20% of a dose of amphotericin B (6, 16). The lack of a clearly defined relationship between serum levels and toxicity or clinical outcome has prevented the development of a rational, systematic approach to amphotericin B therapy.

Many of the problems associated with pharmacokinetic studies of amphotericin B arise from difficulty in obtaining rapid, accurate, and reproducible measurements of drug levels in various biological fluids. Most researchers have used bioassay systems that involve either serial tube dilution or plate diffusion with a variety of indicator organisms, including *Paecilomyces variotii, Candida tropicalis,* and *Saccharomyces cerevisiae* (2-4, 6-8, 17-19). Bioassays, however, vary widely in their accuracy, precision, sensitivity, and specificity, making the comparison of data from different papers difficult.

Several high-pressure liquid chromatographic (HPLC) assays have been reported recently which offer faster and more accurate and reproducible alternatives to bioassays for both pharmacokinetic studies and routine clinical use (9, 10, 13-15, 17, 20). HPLC also offers improved sensitivity and specificity and is easier to standardize than bioassays are. In this report we describe an HPLC method which incorporates an internal standard for the measurement of amphotericin B in serum and a solid-phase extraction procedure for the elimination of potential interference from conjugated bilirubin. We examine the precision, accuracy, recovery, linearity, sensitivity, and specificity of this assay and compare it with a conventional plate diffusion bioassay.

MATERIALS AND METHODS

Reagents and samples. HPLC-grade acetonitrile, methanol, and dimethyl sulfoxide were obtained from Fisher Scientific, Fair Lawn, N.J. Dipotassium EDTA and sodium acetate were purchased from Sigma Chemical Co., St. Louis, Mo., and 1-amino-4-nitronaphthalene was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Unconjugated bilirubin was obtained from the National Bureau of Standards, Washington, D.C., and synthetic taurine-bilirubin conjugate was obtained from Porphyrin Products, Logan, Utah. Stock solutions (20 mg/dl) of the bilirubin preparations were made in human serum albumin (30 g/liter) and diluted with sterile saline before use. The Supelclean C18 solid-phase extraction tubes were purchased from Supelco, Bellefonte, Pa. Amphotericin B type I reference powder and N-acetyl amphotericin B were kindly provided by E. R. Squibb & Sons, Princeton, N.J. A stock solution of each was prepared at a concentration of 1,000 μg/ml in dimethyl sulfoxide-methanol (1:1) and stored at −70°C until needed. Seven reference samples containing 0.2, 0.4, 0.6, 0.8, 1.0, 3.0, and 5.0 μg/ml of amphotericin B were prepared by diluting this stock solution with antimicrobial-activity-free pooled human sera. These samples were used to assess accuracy and intrarun and interrun precision. Forty-nine serum specimens from patients receiving amphotericin B were collected over three months, split into duplicate aliquots, and frozen at −70°C until they were analyzed by both the HPLC assay (using protein precipitation) and the bioassay.

Sample preparation by methanol protein precipitation. A serum sample (200 μl) was combined with 600 μl of methanol (at −20°C) containing the internal standard (1-amino-4-nitronaphthalene; 1.0 μg/ml) and mixed by vortexing for 30 s. After centrifugation at 13,000 × g at room temperature for...
3 min, a 100 μl aliquot of the supernatant was injected into the HPLC system using a U6K injector (Waters Associates, Inc., Milford, Mass.).

Sample preparation by solid-phase extraction. Serum samples containing high concentrations (>3 mg/dl) of conjugated bilirubin were prepared for analysis by solid-phase extraction. A serum sample (200 μl) was prepared as described above, except that N-acetyl amphotericin B (1.0 μg/ml) was substituted as the internal standard. After centrifugation, 500 μl of the supernatant was combined with 500 μl of 0.01 M sodium acetate buffer (adjusted to pH 7.2 with glacial acetic acid). The mixture was transferred to a Supelclean C18 solid-phase extraction column which had been prepared by flushing with two 1.0-ml volumes of acetonitrile followed by two 1.0-ml volumes of sodium acetate buffer. After the sample was loaded, the column was flushed with three to five 1.0-ml volumes of methanol-sodium acetate buffer (1:1), which were discarded. The sample was then eluted with two 0.5-ml volumes of methanol, and a 100-μl aliquot of this final (1.0 ml) eluate was injected into the HPLC system. We were able to reuse the solid-phase extraction columns up to 10 times without any loss of sensitivity or recovery.

Chromatographic conditions. The HPLC system (Waters) consisted of M6000A and M45 pumps connected to a model 720 system controller. The absorbance was monitored with a model 450 variable-wavelength UV detector with 0.04 absorbance units set full scale. Detector response was monitored with a 10-mV OmniscrIBE chart recorder (Houston Instrument Div., Bausch & Lomb, Inc., Austin, Tex.) set at 0.1 in./min. Separation was accomplished at ambient temperature on a μBondapak C18 reverse-phase column (Waters; 3.9 mm by 30 cm). The mobile phase was a mixture of acetonitrile and 0.01 M EDTA (pH 4.2; 40:60 [vol/vol]) delivered at 1.5 ml/min. Other (previously reported) HPLC assays for amphotericin B were performed as described before (10, 14, 17).

Quantitation. A standard curve was constructed by calculating the ratio of the peak height for amphotericin B to the peak height for the internal standard for each of a series of calibration standards. The concentrations of the amphotericin B calibration standards were 0.2, 0.5, 1.0, and 2.0 μg/ml. The peak height ratios for the standards were then plotted against their concentrations, and the amphotericin B concentrations in unknown specimens were calculated by extrapolation from this standard curve.

Bioassay. The microbiological assay used was a modification of the method of Bannatyne et al. (3). Paecilomyces variotii (obtained from J. E. Bennett, Bethesda, Md.) was grown on Emmons Sabouraud dextrose agar slants (GIBCO Laboratories, Lawrence, Mass.) for 5 to 7 days at 35°C. Mature spores from these cultures were harvested by adding 3.0 ml of sterile normal saline to each slant together with five 1.0-ml volumes of methanol-sodium acetate (1.0 ml) eluate). The beads were then rolled gently back and forth across the surface of the slant to dislodge the spores. The spore suspensions from each of the slants were pooled, and the total concentration of spores was determined by counting in a hemacytometer. Antibiotic medium 12 (Difco Laboratories, Detroit, Mich.) was prepared in 250-ml batches, and sufficient spores were added to produce a final concentration of 10^6 spores per ml of agar. This agar was dispensed in 30-ml volumes into sterile petri dishes (150 by 15 mm) and stored at 4°C for up to 5 days. All specimens were assayed in duplicate by punching wells into the seeded agar with a no. 6 (10-mm-diameter) cork borer and by filling the wells with 100-μl volumes of either calibration standards or samples. After incubation for 24 to 32 h at 35°C, the diameters of the zones of inhibited growth were measured to the nearest 0.1 mm with a stereomicroscope (Carl Zeiss, Inc., New York, N.Y.) and plotted (on the abscissa) versus the logarithm of the amphotericin B concentration (on the ordinate). This assay used the same four calibration standards (0.2, 0.5, 1.0, and 2.0 μg/ml) as the HPLC assay.

RESULTS

Specificity, sensitivity, recovery, and linearity. Representative chromatograms of blank serum samples and serum samples spiked with amphotericin B were obtained after methanol protein precipitation (Fig. 1), while a chromatogram of an amphotericin B-spiked serum sample containing N-acetyl amphotericin B as an internal standard was obtained after solid-phase extraction (Fig. 2B). The retention times for amphotericin B and 1-amino-4-nitronaphthalene were 4.9 ± 0.8 and 7.8 ± 1.2 min, respectively. The retention time for N-acetyl amphotericin B was 6.2 ± 1.0 min. A number of antimicrobial agents and amphotericin B analogs were tested and shown not to interfere with this assay (after methanol protein precipitation or solid-phase extraction). These agents included 5-fluorocytosine, ketoconazole, miconazole, econazole, rifampin, tetracycline, vancomycin, tobramycin, gentamicin, amikacin, nystatin, amphotericin B methyl ester, and N-D-ORNithyl amphotericin B methyl ester. Because the metabolites of these drugs were not tested, we
do not know whether the metabolites interfere with the HPLC assays. Because interfering peaks were observed in sera from patients with high total bilirubin levels, pure solutions of conjugated and unconjugated bilirubin were assayed under identical chromatographic conditions to determine the relative contribution of each compound to the interference. The chromatograms of these samples (data not shown) indicate that this interference is due to conjugated bilirubin. Similar interference was also observed with three previously reported HPLC amphotericin B clinical assays (9, 10, 14, 17) of serum from patients with hyperbilirubinemia (data not shown).

The limit of detectability for the HPLC assay under the described conditions was at least 0.04 μg/ml. Since this concentration was less than the usual range of clinical concentrations, its precision and accuracy were not determined. If necessary, sensitivity of the assay may be increased by adjusting the absorbance setting, increasing the injection volume, or concentrating the extract.

Absolute recovery was determined for both HPLC assay methods by comparing the measured concentration of amphotericin B in a spiked serum standard with the measured concentration in a similarly spiked methanol standard. Mean recovery was 92% for both extraction methods.

A composite standard curve was derived from 20 individual standard curves, with the mean peak height ratios ±1 standard deviation plotted against the amphotericin B concentration (Fig. 3). A similar composite standard curve was also constructed for the bioassay (data not shown). Linear regression analysis (5) of these curves indicates excellent linearity for both the HPLC assay and bioassay \((r = 0.998\) and 0.994, respectively). In addition, the linearity of the HPLC assay was examined over a greater range of concentrations by testing serial dilutions of a standard solution of amphotericin B from 10.0 to 0.04 μg/ml and by plotting the resulting measured values against their corresponding expected values. Regression analysis of this curve gave an \(r\) of 0.999, which indicates excellent linearity over a range of concentrations both much less and much greater than those in the usual therapeutic range.

Precision. To define intrarun and interrun precision, we analyzed each of the seven reference samples 10 times in a single run (Table 1) and 10 times in separate runs (Table 2) by the HPLC assay and the bioassay; both protein precipitation

![Graph](http://aac.asm.org/)

**FIG. 3.** Composite standard curve derived from 20 individual standard curves, with the mean peak height ratios ±1 standard deviation plotted against the amphotericin B concentration.

**TABLE 1.** Comparison of HPLC assay and bioassay for intrarun precision

<table>
<thead>
<tr>
<th>Reference concn of amphotericin B (μg/ml)</th>
<th>HPLC assay* (protein precipitation)</th>
<th>HPLC assay* (solid-phase extraction)</th>
<th>Bioassay (agar well diffusion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean* (μg/ml)</td>
<td>CV (%)</td>
<td>Mean* (μg/ml)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>0.2</td>
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<td>0.21 7.62</td>
<td>0.21 4.76</td>
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<td>0.4</td>
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<td>0.34 8.82</td>
</tr>
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<td>0.6</td>
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<td>0.8</td>
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</tr>
<tr>
<td>1.0</td>
<td>0.98 5.10</td>
<td>1.06 2.83</td>
<td>0.95 11.58</td>
</tr>
<tr>
<td>3.0</td>
<td>3.07 1.95</td>
<td>2.97 5.39</td>
<td>2.70 16.30</td>
</tr>
<tr>
<td>5.0</td>
<td>5.34 3.93</td>
<td>5.38 5.39</td>
<td>5.83 22.47</td>
</tr>
</tbody>
</table>

* Amphotericin B concentrations were determined by calculating the peak height ratio (the height of the amphotericin B peak divided by that of the internal standard) and extrapolating from a standard curve.

* Represents the mean of 10 determinations on a single run.
and solid-phase extraction were used to prepare samples for the HPLC assay. With either protein precipitation or solid-phase extraction, the HPLC assay was significantly more precise than the bioassay was, both for intrarun precision (coefficients of variation [CVs] of 2.0 to 6.8 and 2.8 to 9.1 versus 4.8 to 22.5%, respectively) and interrun precision (CVs of 4.9 to 10.0 and 3.9 to 19.2 versus 12.3 to 25.0%).

**Accuracy.** Linear regression analysis was used to define accuracy by comparing the experimental values obtained for the amphotericin B reference samples (y) with their theoretical values (x). Random error in the method was determined by examining r and the standard errors of estimate, while the slopes and y-intercepts were used to estimate the proportional and constant errors, respectively (21). The HPLC assay demonstrated a better correlation with the theoretical values than the bioassay did (r = 0.9995 versus 0.9905). The difference between the methods is more obvious when the standard error of estimate of the HPLC assay (0.063) is compared with that of the bioassay (0.217). Based on this analysis, the bioassay has over three times the random error of the HPLC method.

The equation of the regression line for the HPLC assay was y = 1.064x - 0.033. The slope was not significantly different from 1.0 (P > 0.1), nor was the y-intercept significantly different from 0.0 (P > 0.1) by the Student’s r test, which indicates that there are no significant proportional or constant errors in the method. In contrast, the equation of the line for the bioassay was y = 0.812x + 0.106. Although the y-intercept is significantly different from 0 (P < 0.05), the small positive constant error has little clinical relevance. However, the value of the slope for the bioassay indicates a significant (P < 0.02) proportional error, which could cause clinical problems in interpreting serum levels greater than 2.0 µg/ml.

**Comparative study of HPLC assay and bioassay.** In a direct comparative study, 49 serum specimens from patients receiving amphotericin B were assayed by both the HPLC method (with protein precipitation) and the bioassay. The concentrations determined by the two methods were compared with each other by linear regression analysis (Fig. 4); r was 0.942, and the equation of the line was y = 0.664x + 0.188. The y-intercept of this curve shows a positive constant error similar to that found in the curve for the bioassay alone. Of greater importance is the slope, which indicates that the bioassay significantly (P < 0.01) underestimated the concentration of amphotericin B relative to the HPLC assay.

**DISCUSSION**

To be useful for both pharmacokinetic studies and routine clinical purposes, a drug assay must be simple and rapid as well as precise, accurate, sensitive, specific, and reproducible. Bioassays for amphotericin B, which have been available for some time, may be adequate for routine clinical monitoring and certain pharmacological investigations (1–6, 8). However, their incubation times range from 24 to 36 h, and interference from other antifungal agents or from natural antifungal activity in some human sera is a common problem. In addition, our data suggest that bioassays are less precise throughout the normal range of clinical serum concentrations (8, 18) and less accurate at higher concentrations of amphotericin B. The lack of accuracy at higher concentrations may arise from the proportional relationship between the diameters of the zones of inhibition and the log of the drug concentrations. Depending on the susceptibility of the indicator organism to the drug, the standard curve may become increasingly nonlinear as the drug concentration increases.

Although radiometric bioassays for amphotericin B (7, 11) appear sensitive, accurate, and precise, they require expensive equipment (BACTEC) or facilities for handling radioactive compounds. Furthermore, the reagents are costly and have a relatively short shelf life (7), and test results are affected by 5-fluorocytosine and other antifungal agents (11). In addition, because of deviations from linearity in the standard curve, specimens with amphotericin B concentrations greater than 2.0 µg/ml must be diluted.

Several HPLC procedures for measuring amphotericin B have been developed to overcome the problems inherent in other techniques (9, 10, 13–15, 17), but only two use an internal standard (10, 14). The use of an internal standard compensates for the technical variations which may occur during extraction, injection, or other chromatographic ma-

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**TABLE 2. Comparison of HPLC assay and bioassay for interrun precision**

<table>
<thead>
<tr>
<th>Reference concn of amphotericin B (µg/ml)</th>
<th>HPLC assay* (protein precipitation)</th>
<th>HPLC assay* (solid-phase extraction)</th>
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<td>Mean (µg/ml)</td>
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</tr>
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<tr>
<td>0.2</td>
<td>0.20</td>
<td>10.00</td>
<td>0.26</td>
</tr>
<tr>
<td>0.4</td>
<td>0.37</td>
<td>8.11</td>
<td>0.44</td>
</tr>
<tr>
<td>0.6</td>
<td>0.60</td>
<td>5.00</td>
<td>0.59</td>
</tr>
<tr>
<td>0.8</td>
<td>0.81</td>
<td>4.94</td>
<td>0.80</td>
</tr>
<tr>
<td>1.0</td>
<td>0.98</td>
<td>5.10</td>
<td>1.02</td>
</tr>
<tr>
<td>3.0</td>
<td>3.16</td>
<td>5.06</td>
<td>2.83</td>
</tr>
<tr>
<td>5.0</td>
<td>4.97</td>
<td>7.65</td>
<td>4.77</td>
</tr>
</tbody>
</table>

* Concentrations calculated from the peak height ratio.

* Represents the mean of 10 determinations on 10 separate runs.
jugates readily obtainable, absorbs UV light well at both 382 and 406 nm, is readily soluble in the methanol used for the extraction, and has been used successfully as an internal standard in an HPLC assay of a similar polyene which also used methanol protein precipitation (12). However, we found it necessary to use N-acetyl amphotericin B as an internal standard in the solid-phase extraction assay because 1-amino-4-nitro-naphthalene was retained poorly on the extraction column.

We chose an isocratic mobile phase for the assay to simplify the procedure and to permit the use of simpler, less expensive HPLC equipment. Although Mayhew et al. (14) report that variations in the recovery of amphotericin B are linked to the use of a guard column or precolumn, we employed a 3.0-cm precolumn (Waters) filled with MuBondapak C18/Corsasil and noted no effect on the analytical yield of amphotericin B. Our recovery of amphotericin B from serum samples was greater than 90% over the entire range of concentrations for which the assay was linear. We are unable to account for the variations in recovery reported by other investigators: 53 to 61% (14), 75 to 80% (10), and 98.6 to 100% (17), although the extraction procedures used were virtually identical. We routinely use a sample size of 200 µl, but have found that sample volumes as low as 50 µl are adequate for this assay. The use of small sample sizes (such as 50 µl) is important for defining the pharmacokinetic properties of amphotericin B in pediatric populations.

Although no exogenous compounds were shown to interfere with this assay, we encountered interfering peaks in the serum of patients whose direct bilirubin was greater than 3.0 mg/dl. Our chromatographic analysis of samples containing a pure conjugated and pure unconjugated bilirubin suggests that the conjugates of bilirubin in human serum account for the majority of this interference. This has not been reported previously, although Craven et al. (6) noted that they were unable to measure amphotericin B in bile by HPLC assay due to interfering peaks. The solid-phase extraction procedure we describe effectively eliminates these bilirubin conjugates from the sample (Fig. 2) while providing good recovery (>90%) for both amphotericin B and N-acetyl amphotericin B. The elimination of these peaks is particularly important in the potential use of this assay for pharmacokinetic studies, since the excretion of amphotericin B depends, at least in part, on normal hepatic function (6, 16). Although the solid-phase extraction procedure is more time-consuming than the simple protein precipitation method is, this should not affect the overall utility of the assay for the clinical laboratory, since our experience has been that less than 5% of specimens submitted for amphotericin B assays exhibit these interfering peaks. Otherwise, the HPLC assay method we describe is specific, sensitive, accurate, and precise. This assay should prove useful in pharmacokinetic studies and has the added advantages of simplicity and speed for routine use in the clinical laboratory.

ACKNOWLEDGMENTS

We thank John Bennett for the culture of P. variotii, Michael Barza for sharing his experience, Kwok-Ming Chan for providing the pure conjugated and unconjugated bilirubin samples, and E. R. Squibb & Sons for providing the amphotericin B and N-acetyl amphotericin B used in these studies.

LITERATURE CITED