Quantitative Extraction of Amphotericin B from Serum and Its Determination by High-Pressure Liquid Chromatography

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Therapeutic concentrations of amphotericin B in serum were measured by reversed-phase liquid chromatography with detection at 386 nm. Complete recovery of the drug and the internal standard from a 1-ml serum sample was achieved by pretreating the sample with guanidine hydrochloride and extracting it with a disposable reversed-phase phenyl extraction column. The method was sensitive to 0.005 μg of amphotericin B per ml and linear to at least 5 μg/ml. Coefficients of variation were 4.8% within-run and 6.3% between-run.

Amphotericin B is an antibiotic widely used to treat systemic mycoses (10). Its use is restricted to life-threatening infections, however, because of its severe toxicity, which is usually encountered with therapeutic doses of the drug. Amphotericin B therapy therefore involves a balancing of toxic effects against antifungal activity.

Until recently, lack of a quick, accurate method for assaying the drug in biological fluids hampered attempts at therapeutic monitoring of the drug. In 1977, Nilsson-Ehle and colleagues (8) described a liquid chromatographic assay suitable for clinical use. This method provided the speed, simplicity, and precision which microbiological assays lack. Two subsequent chromatographic assays (6, 7) incorporated internal standards (ISTD) to correct for unpredictable variations inherent in a nonstandardized procedure. None of the above methods involved actual extraction of the drug before chromatography, however, but involved instead precipitation of protein in the specimen by the addition of methanol or acetonitrile. After centrifugation, supernatants were injected into the chromatograph. There are at least two deficiencies in the protein precipitation technique (5). First, precipitation of protein is slow and incomplete. The supernatant contains some protein, as well as other nonprecipitating material, which can clog chromatographic equipment and interfere with the analysis. Second, some of the drug may remain bound to protein and precipitate with it, especially if the drug is relatively hydrophobic, as is amphotericin B. Indeed, Mayhew et al. (7) reported recovering only 53 to 61% of the amphotericin B added to human plasma and suggested that the rest of the drug remained bound to protein.

In this paper I describe a liquid chromatographic assay for amphotericin B which uses both an ISTD and quantitative extraction of the drug from biological fluids.

MATERIALS AND METHODS

Principle. Amphotericin B and an ISTD were extracted from the specimen with a disposable reversed-phase phenyl extraction column. A portion of the extract was injected into the chromatograph, where it was analyzed by standard liquid chromatographic techniques.

ISTD. Disperse yellow dye (5 mg; Sigma Chemical Co., St. Louis, Mo.) was dissolved in 1 ml of methanol. Working ISTD solution was made by diluting the 5-mg/ml methanol solution to 50 μg/ml in 1 M sodium acetate (pH 5.0) containing 5% calf serum to maintain ISTD solubility.

Amphotericin B standard solution. Amphotericin B powder (5 mg) was dissolved in 5 ml of dimethyl sulfoxide (DMSO). This was then mixed with 45 ml of 1% aqueous sodium dodecyl sulfate (SDS). This stock solution (100 μg/ml) was stored at −70°C. To make the working standard solution (1 μg/ml), I combined 1 ml of stock solution, 1.5 ml of 1% SDS, and 97.5 ml of water (total volume, 100 ml). This solution was stored at 1-ml aliquots at −70°C.

Extraction of samples. Samples (serum, aqueous standard, cerebrospinal fluid [CSF], etc.) (1 ml) were combined with 200 μl of ISTD solution and 2 ml of 6 M guanidine hydrochloride. After brief mixing, each sample was passed immediately through a separate, disposable phenyl extraction column (“Bond-Elut” PH, catalog no. 608101; Analytichem International, Harbor City, Calif.). Before analysis, the column was washed with one filling of methanol and then one filling of water. The sample was applied and allowed to run through, and the column was rinsed with another filling of water and allowed to drain. The drug and the ISTD were then eluted immediately from the column into a small test tube with 500 μl of acetonitrile-EDTA (prepared by combining 75 ml of acetonitrile with 25 ml of 5 mM disodium EDTA). SDS (1%) solution (200 μl) was mixed with the extract. Because of the instability of amphotericin B in the specimen after mixing with guanidine, the above steps were completed for one sample before the next sample was extracted. Amphotericin B was very stable in the final extract after SDS was added. For calibration, a 1-ml aliquot of the amphotericin B working standard solution was thawed and immediately extracted as described above.

Chromatography. Chromatography was isocratic, with a solvent consisting of acetonitrile, methanol, and 5 mM EDTA (disodium salt) (187:449:354 [vol/vol]). Analyses were performed with a Series 2/2 pump and an LC-6ST Detector-Oven (The Perkin-Elmer Corp., Norwalk, Conn.) and with a Chromatopac C-R1A data processor (Shimadzu Scientific Instruments, Columbia, Md.) A column (15 cm by 4.6 mm [internal diameter]) packed with 5 μm Ultrasound ODS (model 256-06; Altex, Berkeley, Calif.) was used. It was maintained at 50°C. The mobile-phase flow rate was 1.5 ml/min. The absorbance of the effluent stream was monitored at 386 nm. Ordinarily, 40 μl of extract was injected. Extracts from samples found to contain less than 0.05 μg of amphotericin B per ml were concentrated about fourfold by evaporation at room temperature under a stream of air and reinjected. The concentration of drug was calculated by comparing the ratio of peak areas of drug to peak areas of ISTD given by the unknown with the ratio given by the amphotericin B standard solution (1 μg/ml).
Stability studies. The stability of amphotericin B in solution was examined under various conditions. (i) Solutions in DMSO were stored at room temperature and at 4°C. Aliquots were diluted 1:100 with water and injected immediately into the chromatograph. (ii) Aqueous solutions were stored at room temperature and at 4, −20, and −70°C. After various time intervals, aliquots were injected into the chromatograph. (iii) Solutions in the chromatographic mobile phase were stored at room temperature, and aliquots were injected after various time intervals. (iv) Sera to which known amounts of amphotericin B had been added were stored at room temperature and at 4, −20, and −70°C. Aliquots were extracted and analyzed after various time intervals. (v) Other sera containing amphotericin B were combined with the ISTD and guanidine hydrochloride as for the extraction procedure described above and allowed to stand at room temperature for 1 h before extraction and analysis. (vi) Serum extracts in acetonitrile-EDTA but not including SDS (see above) were allowed to remain at room temperature for 30 min before being injected into the chromatograph. (vii) Extracts including added SDS were allowed to remain at room temperature for 3 days before being injected. (viii) Solutions of amphotericin B and the ISTD in 0.25 or 1% SDS were stored capped at room temperature or 4°C for 14 days. Aliquots were injected daily into the chromatograph. In all these experiments, peak areas for amphotericin B, the ISTD, and early eluting peaks (impurities in, or degradation products of, amphotericin B) were measured.

Statistical parameters. Within-run precision was estimated by adding amphotericin B to 10 blank sera to a concentration of 0.036 μg/ml. This concentration was chosen to assess reproducibility of the method at low levels of the drug. These sera were then extracted and analyzed in one run. Between-run precision was estimated by adding the drug to calf serum to a concentration of 2.6 μg/ml. A total of 29 aliquots, stored at −70°C, were analyzed daily as controls along with routine clinical specimens. The linearity of the method in the therapeutic range was tested by preparing solutions of drug in calf serum at concentrations of 0.05, 0.25, 1, 2, 3, 4, and 5 μg/ml. Each solution was extracted and analyzed in duplicate during a single run. Results were plotted as the concentration of drug added (abscissa, x) versus the concentration measured (ordinate, y). The slope, y intercept, and correlation coefficient were calculated by standard statistical methods.

Bioassay. A limited number of patient samples (serum or CSF) were analyzed both by chromatography and by a turbidimetric bioassay based on the method of Clayton (3). In this assay, 1 μl of specimen is diluted serially into 1-ml portions of antibiotic medium 3 (Difco Laboratories, Detroit, Mich.). Each tube is then inoculated with Candida albicans and incubated at 35°C overnight. The dilution factor of the highest dilution in which no growth is visible is multiplied by the MIC for the culture (typically 0.04 μg/ml) to obtain the concentration of drug in the specimen.

Interference. Unusual portions of 64 sera received for general clinical laboratory testing were carried through the amphotericin B determination. Sera examined were from patients known to be receiving cardioactive drugs (digoxin, procainamide, lidocaine, and quinidine), anticonvulsant drugs (phenobarbital, phenytoin, primidone, and carbamazepine), aminoglycoside agents (amikacin, gentamicin, and tobramycin), and antifungal agents (5-fluorocytosine and ketoconazole). Chromatograms were examined for potentially interfering peaks.

RESULTS

Typical chromatograms are shown in Fig. 1. Generally, early peaks (not numbered) increased with bilirubin concentration. Very icteric specimens sometimes gave peaks overlapping that of the ISTD. Hemolysis and lipemia had no demonstrable effect on the assay, and no interfering drugs were encountered.

The sensitivity of the method, expressed as that concentration of amphotericin B needed to produce a chromatographic peak twice the height of the base-line noise, was ca. 0.005 μg/ml (extract concentrated fourfold as described above). Coefficients of variation were 4.8% within-run (mean, 0.036 μg/ml; standard deviation, 0.00175; n, 10) and 6.3% between-run (mean, 2.62 μg/ml; standard deviation, 0.16; n, 29). The plot of linearity data (not shown) had a slope of 1.04, a y intercept of −0.016 (not significantly different from 0), and a correlation coefficient of 0.9998. The method was therefore linear from at least 0.050 to 5 μg/ml. Linearity was not tested outside this range. Recovery of both amphotericin B and the ISTD from serum was complete with the reversed-phase phenyl extraction columns. Peak areas from water or from serum were statistically identical. Neither drug nor ISTD could be detected in extracted sample residues after a repeat extraction.

Because of the instability of amphotericin B in solution, both reported elsewhere (1) and encountered in this laboratory, the stability of the drug was tested under many conditions. The amphotericin B concentration in serum was found to decrease with a half-life of 21 h at room temperature and a half-life of 20 days at 4°C. After an initial loss of about 5%, sera frozen at −70°C showed no further decrease. Solutions in DMSO showed noticeable degradation after a few hours at either room temperature or 4°C. Serum samples mixed with guanidine for extraction (see above) showed more rapid degradation of the drug than did serum samples alone. The half-life in guanidine was estimated to be 1.5 h at room temperature. Serum extracts in acetonitrile-EDTA also lost amphotericin B rapidly. In contrast, solutions of amphotericin B in 1% SDS showed no detectable loss after 14 days at room temperature. Solutions with less SDS (0.25%) showed slight degradation after 3 days at room temperature but none at 4°C. The addition of 200 μl of 1% SDS to the final sample extract (in 500 μl of acetonitrile-EDTA) preserved the drug in the extract for several days at room temperature.

Table 1 shows the comparison between bioassay and chromatographic assay results.

As of this date (May 1984), the chromatographic assay has been used to determine amphotericin B levels in 119 patient specimens, including serum, CSF, and vitreous humor. Levels in serum and CSF ranged from 0.04 to 3.6 μg/ml, with a combined mean of 0.61 μg/ml.

DISCUSSION

The fact that no interferences other than severe icterus were encountered is not surprising. Amphotericin B is intensely yellow in color, absorbing light at 386 and 409 nm (1). In contrast, most drugs do not absorb light except in the UV region. Amphotericin B is also retained on reversed-phase chromatographic columns much more strongly than most drugs. To interfere with the assay, a substance would have to absorb light at 386 nm and be strongly retained on the column. Few drugs fall into both categories.

Details of the protocol. The protocol, although simple, involved several details which required strict adherence for
in DMSO, it was not well preserved in this solvent. Consequently, solutions in DMSO were immediately mixed with aqueous SDS, which was found to confer greatly increased stability. The drug powder could not be dissolved directly in SDS or sodium deoxycholate. The stability of amphotericin B in the presence of detergents and its instability in other solutions suggested the use of a standard dissolved in SDS rather than in serum. The amount of SDS (0.25 mg) in 1 ml of working standard solution did not interfere with the extraction, but larger amounts (over 0.5 mg) caused incomplete recovery of the ISTD. As discussed above, the addition of SDS to the sample extract increased its stability. SDS also increased by about 30 s the retention time of amphotericin B on the analytical column and sharpened the peak, both presumably by an ion-pairing mechanism.

Use of guanidine. Guanidine hydrochloride was needed to effect complete recovery of both amphotericin B and the ISTD. Urea, another common denaturant, could not be substituted for guanidine. With guanidine present, complete recovery was obtained at pH 5 but not at pH 8.5. The necessity of using a strong protein denaturant such as guanidine in the extraction can be explained by the fact that amphotericin B is strongly bound to serum proteins (2) and must be released before extraction.

The stability of the ISTD in guanidine was good in contrast to that of amphotericin B. The problem this creates can be circumvented by keeping to 5 min or less the time the specimen is exposed to guanidine. If this is done, loss of amphotericin B is less than 4% (based on its life-half of 1.5 h in guanidine).

Choice of extraction column. A phenyl, rather than the more commonly used octadecysilyl (C18), extraction column was necessary for complete extraction of the drug and the ISTD. Apparently, the aromatic phenyl groups of the phenyl column have higher affinity than the aliphatic octadecyl groups of the octadecysilyl column for both the conjugated polyene of amphotericin B and the fused aromatic rings of the ISTD.

Importance of EDTA. Amphotericin B could not be released quantitatively from the extraction column by elution with large volumes of methanol, acetonitrile, or even DMSO. A variable, usually large amount of drug could be recovered by elution of the column with a combination of acetonitrile and aqueous EDTA. This finding is consistent with the observation of Nilsson-Ehle et al. (8) that amphotericin B peaks eluting from their C18 column were neither quantitative nor reproducible in the absence of EDTA. EDTA was therefore included in the mobile phase for the analytical column also. The mechanism of the EDTA effect is unknown. Citrate, another metal ion chelator, had no such effect.

Mobile-phase composition. The composition of the mobile phase (acetonitrile-methanol-EDTA) was chosen so that amphotericin B and the ISTD would move away from each other and from various interfering peaks endogenous to serum. The retention time of amphotericin B was greatly influenced by solvent composition. In acetonitrile-EDTA, for example, it eluted well before the ISTD (see Fig. 1).

Choice of wavelength. The absorption spectrum of amphotericin B has sharp maxima at 409, 386, 367, and 349 nm in aqueous ethanol (1). Nilsson-Ehle et al. (8) used the 409-nm peak for detection because it is the most intense of the four and was found to be least subject to interference in hemolyzed specimens (8). The use of 386 nm in the method described in the present paper was a second choice. The LC-6ST Detector-Oven did not have a visible light source, and

**TABLE 1. Comparison between chromatographic assay and bioassay results**

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Amt of drug (µg/ml) detected by:</th>
<th>Bioassay</th>
<th>Chromatographic assay</th>
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</tr>
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<tr>
<td>CSF</td>
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</table>

* Detection limits depended on the volume of specimen available.

FIG. 1. Chromatograms of the standard (1 µg of amphotericin B per ml) (A) and patient serum (0.68 µg of amphotericin B per ml) (B). Peaks at 4.89 and 6.31 min represent the ISTD and amphotericin B, respectively.
its deuterium (UV) lamp provided insufficient energy at 409 nm. Because the 386-nm peak of amphotericin B is only slightly less intense than the 409-nm peak and because my method included an extraction step which eliminated interference caused by hemolysis (8), monitoring at 386 nm was satisfactory. The ISTD has a broad absorption band centered at 416 nm but extending from 374 to 455 nm (band width at half maximum). It can therefore be monitored at 386 or 409 nm. Correlation between the bioassay and the chromatographic assay (Table 1) was poor, as expected. This can be attributed to the imprecision inherent in any tube dilution method. This imprecision was the primary incentive for the development of the chromatographic assay.

It is interesting to consider how the observed chemical behavior of amphotericin B may help to explain its pharmaco kinetic behavior. The stability of amphotericin B in the presence of detergents is well documented (1, 10). The protective effect of SDS was striking in view of the instability of the drug in DMSO, aqueous acetonitrile, and serum. Apparently, either the drug bound in detergent micelles is inaccessible to more polar molecules which can react with it or the drug is “frozen” into a sterically nonreactive configuration (11). This may explain why the drug was found to be much less stable in serum mixed with guanidine than in serum alone. Guanidine presumably released it from serum protein to which it was bound and rendered it more vulnerable to chemical attack. It is not likely that guanidine itself reacted with the drug. This behavior also suggests an explanation for some of the reported peculiar phar macokinetics of amphotericin B (2, 4, 9). After a course of therapy with amphotericin B, the level of the drug in plasma decreases very slowly, with a half-life of about 2 weeks. Most of the drug is neither excreted in the urine or feces nor metabolized enzymatically by the liver but simply “disappears.” Atkinson and Bennett (2) speculate that the drug is stored in the plasma membranes of muscle and skin cells and is slowly released into the circulation. Because it is stabilized in detergent micelles and phospholipid vesicles (11), it may similarly be stabilized in lipid bilayers. Also, because it is destroyed nonenzymatically in aqueous solutions, it may similarly be destroyed in the bloodstream.

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LITERATURE CITED