# High-Performance Liquid Chromatographic Determination of Amphotericin B in a Liposomal Pharmaceutical Product and Validation of the Assay

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## Abstract

**A validated high-performance liquid chromatographic method is presented to quantitate amphotericin B (AB) in a liposomal pharmaceutical formulation. The analysis is based on the chromatographic separation of AB and 1-amino-4-nitronaphthalene (the internal standard) on a $C_{18}$ µBondapac reversed-phase column with a mobile phase consisting of a mixture of acetonitrile and 0.02M ethylenediamine tetra-acetic acid disodium salt at pH 5.0 (45:55, v/v). The chromatographic analysis time is less than 10 min, and the validation of the assay shows that it is selective, accurate, and linear for the concentration range of 2.50 to 7.50 µg/mL with a detection limit of 0.00500 µg/mL. The within-day and between-day relative standard deviation values are 1.26% ($n$ = 18) and 1.25% ($n$ = 8), respectively. The method described conforms to the validation of compendial methods used for finished pharmaceutical products in general and offers a reliable, quick, and cost-effective procedure for examining the consistency or quality-control analysis of AB in liposomal products. It can also be applied for the determination of AB in other nonliposomal lipid-based drug delivery systems that are on the market.**

## Introduction

Amphotericin B (AB), a macrolide antibiotic isolated from *Streptomyces nodusus*, remains the drug of choice for the treatment of invasive fungal infections. However, its use after intravenous administration of its conventional dosage form, which is a micellar dispersion with sodium deoxycholate (Fungizone, Bristol-Myers Squibb, Princeton, NJ), is associated with severe, dose-limiting, acute, and chronic toxicity (1,2). Several lipid-based drug delivery systems such as liposomes, phospholipid, and cholesterol complexes have been developed in order to reduce the toxicity, improve the therapeutic index, and provide the solubilization of AB by incorporating it into suitable lipid carriers (2–5). AmBisome (NeXtar Pharmaceuticals, San Dimas, CA) was one of the first commercially available liposomal pharmaceutical products and is still used today in clinics internationally. It is a lyophilized formulation of AB incorporated into small unilamellar vesicles having special pharmaceutical characteristics (5,6). At present, no individual pharmacopoeial monographs for the liposomal or lipid-based formulations of AB exist (7,8), but it is essential to use well-established and validated analytical methods during the quality-control evaluation and licensing procedure of AB formulations. Although several high-performance liquid chromatographic (HPLC) methods have been reported for quantitating AB in biological fluids and tissues (9–21), there is no data concerning the quantitative analysis of AB in liposomal formulations or the application of these methods to liposomal or lipid-based formulations of AB that have also fulfilled the requirements of analytical method validation.

Consequently, the purpose of this study was to develop a rapid and simple HPLC method for the determination of AB in a finished liposomal pharmaceutical product such as AmBisome and use it in uniformity testing or determining AB content. In this respect, a modified HPLC method was developed (22) by evaluating previously published HPLC assays for AB quantitation from biological fluids (9–21). A validation protocol has been established according to the analytical parameters described in the analytical methods validation section for finished pharmaceutical products in *The United States Pharmacopoeia—The National Formulary* (USP XXIII-NF XVIII) (8) and other previously published procedures (23,24). This protocol was established because there was no individual specification or definition found in literature for liposomal or lipid-based parenteral drug delivery systems (7,8).

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## Experimental

### Materials

HPLC-grade acetonitrile, methanol (MeOH), and analytical-grade dimethylsulfoxide (DMSO), all purchased from E. Merck (Darmstadt, Germany), were used in this study. The water was purified with a Milli-Q system (Millipore, Milford, MA). AB (Bristol-Myers Squibb, Princeton, NJ) and AmBisome were kindly provided by Er-Kim Drug Inc. (Istanbul, Turkey). Distearoyl phosphatidylglyserol, cholesterol, and hydrogenated soybean phosphatidylcholine (each having high purity) were purchased from Sigma Chemicals (Deisenhofen, Germany) and Lipoid GmbH (Ludwigshafen, Germany). 1-Amino-4-nitronaphthalene was purchased from Aldrich (Steinheim, Germany). All of the other substances were of analytical reagent grade.

### Apparatus and conditions

The liquid chromatographic (LC) system (Shimadzu, Kyoto, Japan) that was used consisted of a model LC-6A pump, an ultraviolet–visible (UV–vis) SPD-6AV detector, and a Chromatopac CR3A integrator for recording and integrating with an attenuation range set between 7 and 10, which corresponded to the recorder attentuation of 128–1024 mV. The chromatographic signal was monitored at 407-nm wavelength based on the UV spectrum of AB and AmBisome in the mobile phase and recorded by a UV–vis 16 spectrophotometer (Shimadzu) with 0.1 absorbance units at full scale (AUFS). The chromatographic separation was performed at ambient temperature on a reversed-phase $C_{18}$ µBondapac column (300- × 4.6-mm i.d., 5-µm particle size) (Waters, Milford, MA). A Rheodyne model 7125 injection valve (Rheodyne, Cotati, CA) with a 100-µL capacity loop was used for sample injection. The elution was established with a mobile phase having a composition of acetonitrile and 0.02M ethylenediamine tetra-acetic acid disodium salt ($Na_2EDTA$) at pH 5.0 (45:55, v/v) and a flow rate of 1.0 mL/min. Prior to use, the mobile phase was filtered under vacuum through 0.45-µm nucleopore membrane filters (Costar, Bodenheim, Germany) and degassed using a sonication bath for at least 30 min. In order to control the HPLC runs, 1-amino-4-nitronaphthalene was used as an internal standard (IS).

### Standard solutions

Stock solutions of AB (50 µg/mL) and 1-amino-4-nitronaphthalene (50 µg/mL) were prepared separately in a mixture of DMSO–MeOH (1:1, v/v). The working standard solutions for constructing calibration curves and the assay validation covering the range for intended use (50–150%) were obtained by the appropriate dilution of the stock solutions in acetonitrile–0.025M $Na_2EDTA$ at pH 4.2 (60:40, v/v).

### Sample preparation

Samples were prepared individually by using 10 vials of AmBisome. Each vial's content was dissolved in a mixture of DMSO–MeOH (1:1, v/v) and then transferred to a volumetric flask and made up to a volume of 100 mL. The stock solutions containing 50 µg/mL of AB were prepared from the dissolved liposomal products by further dilution with the same solvent mixture. In order to obtain sample solutions containing 5.00 µg/mL of AB and the IS, the IS was added to the aliquots of the AB stock solutions in required amounts and diluted with acetonitrile–0.025M $Na_2EDTA$ at pH 4.2 (60:40, v/v). Each sample was analyzed at triplicate by HPLC for the determination of AB content in AmBisome.

### Assay validation

As indicated by the manufacturer, hydrogenated soybean phosphatidylcholine (213 mg), distearoyl phosphatidylglycerol (84 mg), cholesterol (52 mg), disodium succinate hexahydrate (27 mg), and sucrose (900 mg) were the liposomal ingredients in each of the vials containing AmBisome and 50 mg of AB that was incorporated into the liposomal lipid bilayers.

In order to assess the accuracy of the assay, a synthetic mixture was prepared because a placebo liposomal product was not available. Consequently, for the preparation of the drug-free synthetic mixture (placebo), accurate amounts of the liposomal ingredients as indicated by the manufacturer were weighed and dissolved in DMSO–MeOH (1:1, v/v) by using an ultrasonic bath for 10 min. It was then filtered and diluted to 100 mL in a volumetric flask with the same solvent mixture. Three working standard solutions of AB (having 2.50 µg/mL, 5.00 µg/mL, and 7.50 µg/mL of AB, respectively, and each containing 5.0 µg/mL of the IS) were prepared by spiking AB and the IS into the synthetic mixtures from the stock solutions and diluting them with acetonitrile–0.025M $Na_2EDTA$ at pH 4.2 (60:40, v/v). Six consecutive measurements were performed for each concentration within the same day. The percent recoveries of AB were calculated from the concentrations recovered in the synthetic mixtures divided by the theoretical concentration added to the synthetic mixtures. The individual and mean absolute recovery between 98 and 102% with a relative standard deviation (RSD) of less than or equal to 2% was the criteria for acceptance in the accuracy evaluation.

The precision of the system was examined by taking 6 consecutive measurements of the working standard solution of AB at 5.00 µg/mL and the IS (prepared as described in the Standard solutions section) within the same day. The within-day and between-day precision of the method were tested in terms of repeatability and reproducibility. In repeatability, 3 independent working standard solutions of AB at 3.00 µg/mL, 5.00 µg/mL, and 7.00 µg/mL (representing 60–140% of the calibration range and each having 5.00 µg/mL of the IS) were prepared from the liposomal product of AB, and 6 measurements were performed for each concentration within the same day. Reproducibility of the assay was evaluated by using the 5.00-µg/mL working standard solution of AB and the IS that was also prepared from the liposomal product. This solution was analyzed twice a day at 4 different days. The acceptance criteria for all of the precision measurements was that the RSD value should be no greater than 2%.

The intended application of the procedure was to quantitate AB in a pharmaceutical product, and the range of the working standard solutions for constructing calibration curves and evaluating linearity was kept between 50 and 150% of the theoretical value of AB at 5.00 µg/mL, which also represented the mid-range. Five working standard solutions at 2.50 µg/mL, 3.50 µg/mL, 5.00 µg/mL, 5.50 µg/mL, and 7.50 µg/mL of AB (each containing 5.00 µg/mL of the IS) were prepared on every working day as described in the Standard solutions section. Triplicate measurements were

performed for each concentration, and the calibration curves were constructed by plotting the ratio of the peak area of AB to that of the IS as a function of the nominal concentration of AB in the working standard solutions. The data were statistically evaluated using a nonlinear regression analysis software package called EKKAR4.1, and the equations of the calibration curves were calculated by least-square linear regression analysis.

The sensitivity of the method was determined by using 8 working standard solutions of AB between 0.00500 and 2.50 µg/mL, which were prepared as described in the Standard solutions section. After analysis by HPLC, the concentration that was measured with accuracy and precision was accepted as the limit of quantitation, and the limit of detection was determined at a signal-to-noise ratio of 3:1.

In order to ensure the specificity of the HPLC assay for AB, the drug-free synthetic mixture prepared from the liposomal ingredients was examined by HPLC under the final assay conditions. Additionally, the synthetic mixture spiked with AB, the working standard solution of AB that did not contain IS, and the sample solution prepared from AmBisome were also analyzed. The chromatograms obtained from these analyses were compared for the possibility of interferences.

### Quality control samples

Quality control samples in duplicate at 3 different concentrations—one near the lower limit (4.00 µg/mL), one in the mid-range (5.00 µg/mL), and one in the upper limit (7.00 µg/mL) of the calibration curve (80–140%) and each having 5.00 µg/mL of the IS—were prepared fresh for each run as described in the Standard solutions section. On the day of analysis, the quality control samples were incorporated randomly into the run (such as during the construction of the calibration curves and along with the unknown samples) to ensure the reliability of the method for everyday application. The individual and overall mean percent recovery of AB from the quality control samples were calculated from the peak area ratios of AB to that of the IS as described in the Assay validation section. An RSD value of less than or equal to 2% provided the basis of the acceptance criteria for the run.

### Stability of amphotericin B

In order to assess the stability of AB in the DMSO–MeOH (1:1, v/v) mixture, the aliquots taken from the stock solution of AB as described in the Standard solutions section were stored in closed vials at 4°C and 25°C in the dark and at 25°C exposed to fluorescent light. For the samples withdrawn from each vial at the beginning and at the end of the first, tenth, and thirtieth day, the IS was added and diluted with acetonitrile–0.025M $Na_2EDTA$ at pH 4.2 (60:40, v/v) to the 5.00-µg/mL concentration of AB and the IS. Triplicate measurements were performed by HPLC, and the mean percent of AB that remained was calculated for each sample.

### Preparation and storage conditions

All procedures during the preparation of the stock, standard, and sample solutions and the quality control samples were completed in a darkroom, and all of them were kept at 4°C and wrapped in aluminum foil in order to prevent the degradation of AB.

## Results and Discussion

### Optimization of chromatographic conditions

Mobile phases of different compositions (including ternary mixtures of MeOH–acetonitrile–$Na_2EDTA$ and binary mixtures of acetonitrile–$Na_2EDTA$ and MeOH–$Na_2EDTA$ at different levels of pH and molarity) were reported for the quantitation of AB from biological fluids by HPLC (9–22). Preliminary experiments were conducted to obtain optimum separation in the use of these mobile phases. When ternary mixtures of MeOH–acetonitrile–$Na_2EDTA$ were examined according to Bach (10) and Kan et al. (17), the resolution was not achieved because of the overlapping of the peaks relating to MeOH and the IS. For this reason, binary mixtures of acetonitrile–$Na_2EDTA$ were tested. However, the mobile phase used by Granich et al. (11) that consisted of acetonitrile–0.01M $Na_2EDTA$ at pH 4.2 (40:60, v/v) also demonstrated poor resolution in our experimental conditions. In their study, serum AB concentration was determined after precipitating serum proteins with MeOH, and the retention times reported for AB and 1-amino-4-nitronaphthalene were 4.9 ± 0.8 and 7.8 ± 1.2 min, respectively. In our study, in order to improve the separation, visual inspection of the peak resolution was established by increasing the molarity and pH of $Na_2EDTA$. Thus, a good resolution was obtained with the mobile phase having a composition of acetonitrile–0.02M $Na_2EDTA$ at pH 4.5 (45:55, v/v), but the elution order of AB and the IS was changed. The mobile phase used in this latter case had the same composition reported by Gondal et al (13). They determined the AB concentration in plasma by HPLC after extracting with MeOH, but neither the chromatograms nor the retention times were reported in

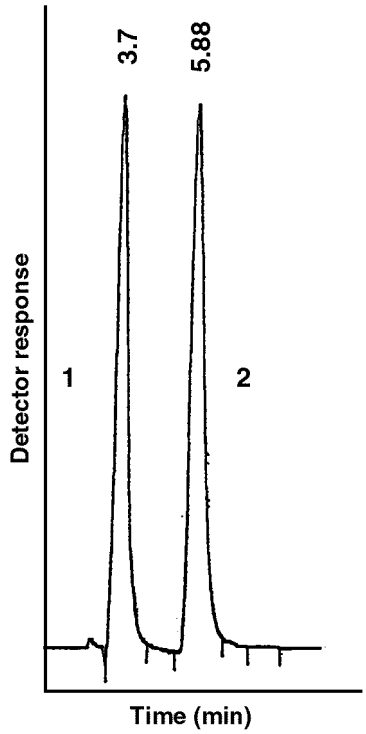


**Figure 1.** Chromatographic separation of the IS (peak 1) and AB (peak 2) in a working standard solution of AB at 5.00 µg/mL.

their study. In our experimental conditions, the pH was further increased, and finally, reproducible separations were achieved by eluting with a mobile phase consisting of acetonitrile–0.02M $Na_2EDTA$ at pH 5.0 (45:55, v/v). During the analysis, the UV detection was performed at 407 nm. This wavelength was also found to be suitable for the detection of the IS. A typical chromatogram that was developed under the final assay conditions after injecting a working standard solution of AB is illustrated in Figure 1. The chromatogram was recorded under the final HPLC assay conditions in the absorbance range of 0.32 AUFS by using the integrator attenuation set at 8, which corresponded to the recorder attenuation of 256 mV. Also, it should be noted that AB was eluted after the IS, and the mean retention times for AB and the IS were 5.84 ± 0.01 min (RSD 0.445%, $n$ = 10) and 3.75 ± 0.02 min (RSD 1.50%, $n$ = 10), respectively, on different occasions.

### Assay validation

The accuracy of the method was ascertained by analyzing the synthetic mixtures spiked with AB at 3 concentrations, representing 50–150% of the calibration range. The results were determined by comparing the measured concentrations to its nominal values and expressed in percent recovery, as shown in Table I. Data on the individual and mean absolute analytical recovery of AB showed that the essential satisfactory recovery between 98.0 and 101% and RSD values of less than or equal to 2% were obtained.

In the precision estimation of the system, the RSD value was found to be 0.404% after 6 consecutive injections of the working standard solution of AB at 5.00 µg/mL. The within-day precision results (repeatability) are shown in Table II, and the RSD value for 18 measurements was 1.26%. The between-day RSD value, calculated from the analysis performed twice a day at 4 different days (reproducibility), was found to be 1.25% ($n$ = 8). These results met the acceptance criterion for precision evaluations and showed that the precision of the system and the method suited the purpose of the analytical method because the RSD values were below 2%.

**Table I. Accuracy Results Obtained for the Analysis of Amphotericin B**

| Concentration (µg/mL) | Added amount (%) | PAR* | Found amount (%) | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| 2.50 | 50 | 0.833 ± 0.001 | 49.0 ± 0.3 | 98.0 ± 0.5 | 1.25 |
| 5.00 | 100 | 1.55 ± 0.004 | 101 ± 0.1 | 101 ± 0.1 | 0.303 |
| 7.50 | 150 | 2.21 ± 0.006 | 150 ± 0.0 | 99.8 ± 0.6 | 0.567 |
| Mean ± standard deviation ($n$ = 18) | | | | 99.7 ± 0.4 | 1.60 |
| 95% Confidence limits | | | 100 > µ > 98.9 | | |

* PAR, peak-area ratio (AB/IS) (mean ± standard deviation, $n$ = 6).
† Calculated from PAR and expressed as the found amount in percent (mean ± standard deviation, $n$ = 6).
‡ RSD, relative standard deviation.

**Table II. Within-Day Precision Results of the HPLC Method**

| Concentration (µg/mL) | Added amount (%) | PAR* | Found amount (%) | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| 3.00 | 60 | 0.991 ± 0.005 | 60.6 ± 0.3 | 101 ± 1.0 | 1.58 |
| 5.00 | 100 | 1.56 ± 0.00 | 99.3 ± 0.2 | 99.3 ± 0.2 | 0.381 |
| 7.00 | 140 | 2.13 ± 0.00 | 139 ± 0.0 | 99.2 ± 0.2 | 0.531 |
| Mean ± standard deviation ($n$ = 18) | | | | 99.8 ± 0.3 | 1.26 |
| 95% Confidence limits | | | | 100 > µ > 99.1 | |

* PAR, peak-area ratio (AB/IS) (mean ± standard deviation, $n$ = 6).
† Calculated from PAR and expressed as the found amount in percent (mean ± standard deviation, $n$ = 6).
‡ RSD, relative standard deviation.

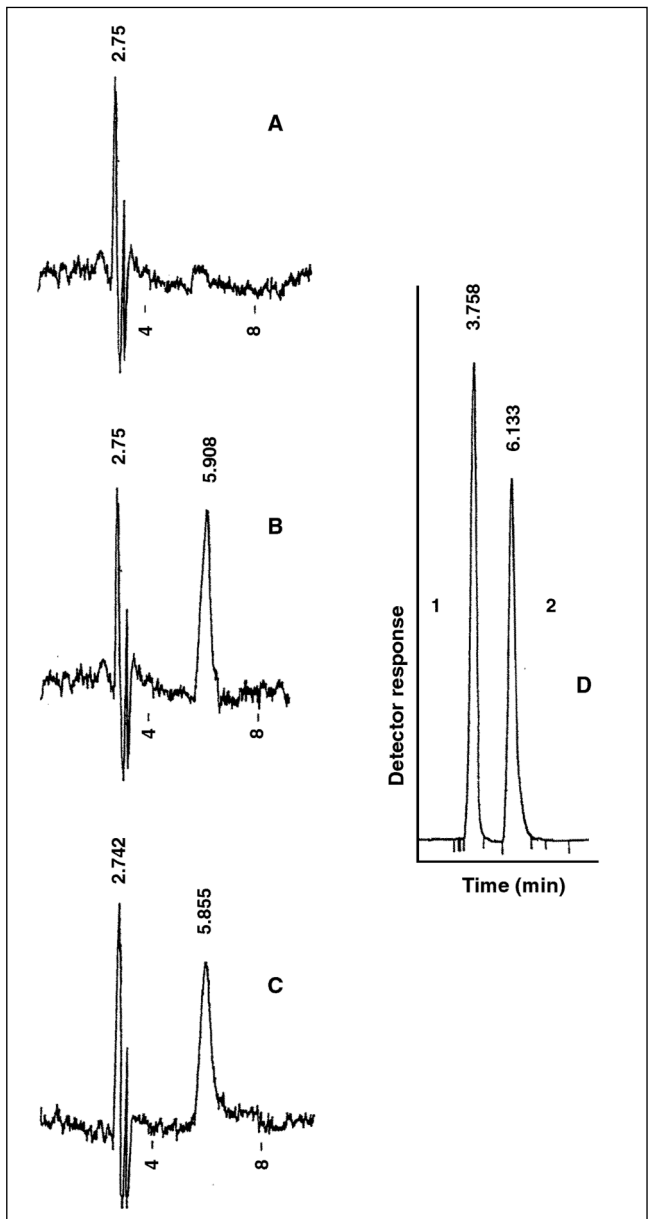


**Figure 2.** Representative chromatograms of a synthetic mixture prepared from the liposomal ingredients (A), a synthetic mixture spiked with AB (0.0100 µg/mL) (B), a working standard solution of AB (0.0100 µg/mL) without IS (C), and a sample solution prepared from AmBisome containing 2.50 µg/mL of AB (D). For Figure 2D, peak 1 is the IS and peak 2 is AB.

The linearity of the method for AB assay was evaluated between the concentration range of 2.50 and 7.50 µg/mL, and the relationship of the peak-area ratio of AB to the IS and the concentration of AB was found to be linear. A typical regression equation for the calibration curve was $y = 0.294x + 0.0571$ with a coefficient of determination of 0.9999, and the intercept was not significantly different from zero ($P = 0.05$). AB calibration curves also displayed good reproducibility at different days, and the mean of the slope, intercept, and coefficient of determination were 0.300 ± 0.00318 (RSD 1.84%, $n = 3$), 0.0394 ± 0.00997 (RSD 43.9%, $n = 3$), and 0.9986 ± 0.000333 (RSD 0.0578%, $n = 3$), respectively. The quantitation limit was the lowest concentration of an analyte in a sample that can be determined with acceptable accuracy and precision under the stated experimental conditions. Although the working standard solution of AB at 2.50 µg/mL also fulfilled the requirements, 8 concentrations examining the working standard solution of AB at 0.500 µg/mL also met the requirements in determining the quantitation limit. The mean measured amount of AB at this level was 0.484 ± 0.003 µg/mL (RSD 1.27%, $n = 6$). The detection limit as defined in the experimental section was 0.00500 µg/mL.

The absence of interference between the peak responses of AB and the IS and the liposomal ingredients such as phospholipids and cholesterol was verified by visual inspection of the chromatograms. The typical chromatograms that were obtained are illustrated in Figure 2. These chromatograms were recorded under the final HPLC assay conditions with different attenuation settings in order to demonstrate the absence of interference because of the liposomal ingredients. The integrator's attenuation setpoints in Figures 2A–2C were selected as 1, which corresponded to the recorder attenuation of 2 mV and the absorbance range of 0.0025 AUFS. The chromatogram in Figure 2D was plotted in the absorbance range of 0.16 AUFS using the integrator attenuation set at 7 and a recorder attenuation of 128 mV. No endogenous peaks were detected at the retention times of interest. There was no significant difference found between the retention time of the working standard solutions of AB and the spiked synthetic mixture. The mean retention time of AB in the spiked synthetic mixture was 5.88 ± 0.01 min (RSD 0.359%, $n = 10$). However, a slight variation in the retention time of AB in the liposomal product (6.11 ± 0.02 min, RSD 0.758%, $n = 10$) was observed without having an influence on its peak area or shape, as demonstrated in Figure 2D. This was attributed to the preparation method of the AB liposomal formulations. To our knowledge, in order to prepare a stable liposomal product with a hydrophobic drug like AB, the drug should first be complexed with a negatively charged phospholipid such as phosphatidylglycerol when dissolving it in organic solvents, then the other phospholipids and lipid ingredients should be added. In this respect, the addition order of AB and the lipid ingredients had special importance during the preparation of its liposomal products. The addition of AB into a synthetic mixture or a preformed placebo liposomal formulation would not allow AB to interact sufficiently with the negatively charged phospholipid present in the medium, as maintained during the preparation steps of a real pharmaceutical liposomal product. Thus, the extent of interaction and the complex formation of AB with the negatively charged phospholipid could be altered or reduced. Consequently, this difference could be the reason for the slight shift observed in the retention time of AB in the liposomal product, although no interfering new peaks dealing with the liposomal ingredients were detected. The synthetic mixtures or placebo formulations that should be necessary during the assay validation procedure of other lipid or cholesterol complexed nonliposomal formulations of AB might also show the same variation because of the complex forming behavior of AB, but this phenomena should be further investigated. For this reason, the liposomal and lipid-based new drug delivery systems of AB and other drugs and the procedures dealing with their analytical assay validation should be well defined by taking their pharmaceutical formulation characteristics into consideration, and their individual monographs should also be included in interna-

**Table III. Within-Day and Between-Day Recovery Data of Amphotericin B from the Quality Control Samples**

| Concentration (µg/mL) | Day 1 Recovery* (%) | Day 2 Recovery* (%) | Day 3 Recovery* (%) | Different days Recovery (%) |
|---|---|---|---|---|
| 4.00 | 99.5 ± 1.4 | 102 ± 0.0 | 102 ± 0.0 | 101 ± 1.0 (1.55) |
| 5.00 | 99.3 ± 0.4 | 101 ± 1.0 | 102 ± 0.0 | 101 ± 1.0 (1.33) |
| 7.00 | 99.3 ± 0.4 | 98.9 ± 0.0 | 98.3 ± 0.3 | 98.8 ± 0.5 (0.569) |
| | 99.4 ± 0.9 (0.974)† | 102 ± 1.0 (1.49)† | 101 ± 2.0 (1.90)† | 101 ± 2.0 (1.56)‡ |

* Calculated from peak-area ratio and expressed as percent recovery (mean ± standard deviation, $n = 2$).
† Calculated from peak-area ratio and expressed as percent recovery (mean ± standard deviation, $n = 6$).
‡ Calculated from peak-area ratio and expressed as percent recovery (mean ± standard deviation, $n = 18$).

**Table IV. Determination of AB Content in AmBisome**

| Vials | Concentration (µg/mL) | PAR* | Recovery (%) |
|---|---|---|---|
| 1 | 5.00 | 1.45 ± 0.03 (0.413) | 97.4 ± 0.3 (0.452) |
| 2 | 5.00 | 1.36 ± 0.00 (0.558) | 88.9 ± 0.0 (0.0649) |
| 3 | 5.00 | 1.52 ± 0.01 (0.761) | 100 ± 0.0 (0.707) |
| 4 | 5.00 | 1.49 ± 0.01 (0.400) | 98.0 ± 0.3 (0.513) |
| 5 | 5.00 | 1.49 ± 0.00 (0.471) | 106 ± 0.0 (0.471) |
| 6 | 5.00 | 1.53 ± 0.01 (0.808) | 101 ± 0.0 (0.805) |
| 7 | 5.00 | 1.54 ± 0.00 (0.226) | 100 ± 0.0 (0.220) |
| 8 | 5.00 | 1.54 ± 0.01 (0.768) | 101 ± 0.0 (0.705) |
| 9 | 5.00 | 1.49 ± 0.01 (0.594) | 100 ± 0.0 (0.591) |
| 10 | 5.00 | 1.54 ± 0.00 (0.366) | 98.0 ± 0.2 (0.360) |
| Mean ± standard deviation, relative standard deviation (%), $n = 10$ | | | 99.3 ± 1.4 (4.30) |
| 95% Confidence limits | | | 102 > µ > 96.2 |

* PAR, peak-area ratio (AB/IS) (mean ± standard deviation, relative standard deviation (%), $n = 3$).
† Calculated from PAR and expressed as percent recovery (mean ± standard deviation, relative standard deviation (%), $n = 3$).

tional pharmacopoeias. In our study, the use of the synthetic mixture that was prepared by dissolving all of the ingredients in organic solvents minimized or eliminated the difference in the retention times of AB in the working standard solutions and the spiked synthetic mixtures. Finally, the method in our study and its validation protocol could also be applied to the quantitative analysis of other nonliposomal formulations of AB.

In this study, the quality control samples at 3 levels were used during the construction of calibration curves in each day and during the application of the assay. They were analyzed in duplicates by the random introduction to the run in order to assess and control the within-day and between-day variations. The results that deal with the analysis of 6 quality control samples at 3 different days are expressed in percent recovery in Table III. As defined in the Experimental section, in all cases the RSD values were found to be within the recommended values by not exceeding 2%. This data also indicated that the difference between the measured and actual concentrations was not significant, and the run dealing with the determination of AB content (as shown in Table IV) was accepted. As mentioned previously, because no individual pharmaceutical monograph dealing with liposomal AB has been found, the general requirements described in USP XXIII-NF XVIII for the content uniformity testing of parenteral products were taken into consideration in order to evaluate the consistency of the liposomal product (8). The requirements stated in the USP for dose uniformity were met as long as the amount of active ingredient in each of the 10 dosage units was within the range of 85.0 to 115.0% of the label claim and the RSD value was less than or equal to 6%. In our study, the percent amount of AB determined in 10 unit vials of AmBisome were between 88.9 and 106% of the claimed value. The individual and overall RSD values were less than 6%. Finally, these results were found to be in accordance with the pharmacopoeial requirements given for parenteral products in USP XXIII-NF XVIII (8).

### Stability of amphotericin B

It has been reported that AB is soluble in DMSO, but it was not well preserved in this solvent (10,25). Consequently, MeOH was added usually for increasing its stability, and binary mixtures of DMSO–MeOH were used for the preparation of stock solutions (9,11,13). At the beginning and over the course of our study, it has been found that AB also decomposed in a DMSO–MeOH mixture over time and when the samples were exposed to light. Because of this finding, special attention was given to the light and temperature sensitivity of AB in this mixture during the storage and preparation of the solutions and samples, and all of the procedures performed before the analyses were carried out in a darkroom. The results of AB stability in DMSO–MeOH mixtures are given in Table V. The effect of light on degradation was found to be rapid, and the degradation observed could be prevented by protecting the samples from light exposure. After one month of storage at 4°C in the dark, no change in AB concentration was detected. These findings could have profound implications for the analysis of AB, because the stock and working solutions that were allowed to stand without light protection or other procedures carried out in light could influence the validation data by the degradation of the drug.

**Table V. Stability of AB in the Stock Solution**

| Time (day) | % Drug remaining* 25°C (in the dark) | 25°C (light exposed) | 4°C (in the dark) |
|---|---|---|---|
| 0 | 101 ± 0.0 (0.571) | 101 ± 0.0 (0.571) | 101 ± 0.0 (0.571) |
| 1 | | 101 ± 1.0 (0.979) | 99.1 ± 0.5 (0.816) |
| 101 ± 1.0 (1.49) | | | |
| 10 | | 95.1 ± 1.1 (1.98) | 77.6 ± 0.2 (0.451) |
| 101 ± 1.0 (1.51) | | | |
| 30 | | 88.9 ± 0.2 (0.454) | 74.5 ± 0.6 (1.51) |

## Conclusion

A simple and rapid HPLC method has been established to determine the concentration of AB in a liposomal pharmaceutical product. The time for chromatographic analysis was less than 10 min per sample and provided an easy approach to evaluate the AB content without applying any extraction procedures. The method was demonstrated to be accurate, precise, and sensitive for the analytical characterization and quantitation of AB without showing interference from the liposomal excipients examined such as phosphaditylcholine, phosphatidylglycerol, and cholesterol. Because these substances are among the main constituents of other lipid-based drug delivery systems for AB, this method can easily be applied to these kinds of formulations such as ABELCET and Amphocil.

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