Liquid Chromatographic Determination of Amphotericin B in Different Pharmaceuticals

LOUIS P. LUE, SUSAN T. HADMAN, and ALES VANCURA
U.S. Food and Drug Administration, Department of Health and Human Services, Office of Regulatory Affairs, Northeast Regional Laboratory, 158-15 Liberty Ave, Jamaica, NY 11433

Amphotericin B (AmB) is one of the most potent antifungal agents and the drug of choice in the treatment of serious fungal infections. A liquid chromatographic (LC) method was developed to determine AmB in pharmaceutical formulations for injection, tissue culture, cream, and lotion. A Bondapak C18 reversed-phase column and a simple mobile phase consisting of acetonitrile–water–acetic acid (40 + 54 + 6, v/v) was used. The flow rate was 1.8 mL/min and the effluent was monitored at 405 nm. The developed LC method uses piroxicam as an internal standard and has a limit of detection of 10 ng/mL, a limit of quantitation of 30 ng/mL, and the assay is linear from 0.01 to 100 µg/mL. AmB and piroxicam elute with retention times of 12.4 and 4.0 min, respectively, and the resolution between AmB and piroxicam was 10.6. In comparison with the official United States Pharmacopeia microbial assay for AmB, this LC method is more rapid, selective, sensitive, and offers positive identification.

Amphotericin B (AmB) is a polyenic antibiotic isolated from actinomycete Streptomyces nodosus. The antifungal activity of AmB consists in binding of AmB to ergosterol present in cell membranes of fungal cells. This binding results in selective destruction of fungal cell membranes and killing of fungal cells. After more than 30 years of clinical use, AmB remains the drug of choice for the treatment of serious systemic fungal infections (1). The wide use of AmB in recent years stems from the dramatic increase in fungal infections due to the lack of immunocompetence caused by chemotherapy and spread of AIDS (1). However, the clinical use of AmB is hampered by the considerable treatment-associated toxicity of the drug, the most serious being nephrotoxicity (2). To reduce the toxicity and to improve the therapeutic index, several lipid-based drug delivery systems such as liposomes, phospholipid, and cholesterol complexes have been developed for AmB (3, 4).

One of the major requirements for AmB premarketing certification is passing the microbial agar diffusion assay (5, 6), which determines the total activity (potency) of AmB by measuring the inhibitory effect of AmB on a sensitive organism, Saccharomyces cerevisiae. However, the microbial assay of AmB has limitations inherent to an assay based on measuring the activity rather than the quantity of the analyzed compound: it lacks the specificity and positive identification of AmB.

Numerous liquid chromatographic (LC) methods have been developed for the analysis of AmB in biological matrices such as plasma, serum, urine, respiratory secretions, different tissues, etc. (7–18). LC on reversed-phase column for AmB quantitation has been used by Margosis and Aszalos (8). However, no internal standard was used to improve the precision in this study. Lambros et al. (15) used natamycin as an internal standard and 2 different UV wavelengths, 383 and 303 nm, for detection of AmB and the internal standard, respectively. Lacroix et al. (13) introduced photodiode array detection of AmB, which eliminated the requirement for 2 detectors configured at 2 different wavelengths. Piroxicam was used as an internal standard by Echevarria et al. (16). The absorbancy wavelength of piroxicam is similar to that of AmB and, therefore, both compounds can be detected at 405 nm. The mobile phases used in the reported methods were mixtures of acetonitrile with aqueous buffers.

The analysis of formulated AmB by LC has not been reported. The purpose of this study was to develop a rapid and simple LC method for the determination of AmB in bulk and finished pharmaceutical products (AmB for injection, tissue culture, and lotion), including lipid-based formulations (AmB cream). This LC assay represents a rapid and accurate alternative to the time-consuming official United States Pharmacopeia (USP) microbial assay method (5).

Experimental

Apparatus

(a) LC system.—Shimadzu SCL-10A system controller, LC-10AS pump, SPD-10 AV UV-visible detector, and SIL-10A auto-injector. E-Z Chrom software was used for data acquisition. The LC column was Waters Bondapak C18 reversed-phase column, 300 × 3.9 mm id, 10 µm packing particle size. Mobile phase was acetonitrile–water–acetic acid (40 + 54 + 6, v/v). The sample was injected in 40 µL, the mobile phase was delivered at 1.8 mL/min, and the detector was set at

Received March 21, 2001. Accepted by JM August 8, 2001.
Corresponding author’s e-mail: llue@ora.fda.gov.
405 nm. Isocratic elution was used throughout and the time of analysis was typically 16 min.

(b) Visiprep solid-phase extraction vacuum manifold.—Used for sample preparation (Supelco, Bellefonte, PA).

Reagents

(a) AmB USP reference standard (RS).—U.S. Pharmacopeial Convention (Rockville, MD).
(b) Piroxicam.—Sigma (St. Louis, MO).
(c) Water (LC grade), acetonitrile, methanol, petroleum ether, and dimethyl sulfoxide (DMSO).—Fluka (St. Louis, MO).
(d) C18 cartridges.—Waters (Milford, MA).
(e) AmB (not formulated).—Sigma; used as “bulk” AmB.
(f) Formulations of AmB (including 3% AmB cream, USP; 3% AmB lotion, USP; Fungizone for injection; and Fungizone for tissue culture).—Bristol Myers Squibb (Cranbury, NJ).

Preparation of Standard Solutions

Low-actinic glassware was used for preparation of the standard and sample solutions. Ten milligrams AmB USP RS was predried as dictated by the USP monograph, accurately weighed, and delivered into a 10 mL volumetric flask. Dimethyl sulfoxide–methanol (1 : 1, v/v) was added up to mark, yielding AmB standard stock solution at 1.0 mg/mL. The standard stock solution was further diluted with mobile phase to 10, 50, 100, 250, and 500 ng/mL and 1, 10, 20, 40, 60, and 100 g/mL for a limit of detection (LOD) and limit of quantitation (LOQ) study.

The internal standard stock solution of piroxicam in acetonitrile was made at a concentration of 1.0 mg/mL, as described above for AmB. Standard stock solutions of AmB and piroxicam were mixed and diluted with mobile phase, as needed.

Sample Preparation

(a) Unformulated AmB.—AmB purchased from Sigma was used as a bulk sample (unformulated AmB), and predried as the USP standard. Fifty milligrams of the bulk sample were accurately weighed, dissolved in 10.0 mL of DMSO in a 50 mL volumetric flask, and 10.0 mL methanol was added. Subsequently, water was added to the mark, yielding 1.0 mg/mL AmB solution, designated as bulk stock solution. AmB bulk stock solution (3.0 mL) and 10.0 mL piroxicam stock solution were transferred into a 100 mL volumetric flask and the flask was filled with mobile phase to the mark, yielding a solution containing AmB at 30 g/mL and piroxicam at 100 g/mL.

(b) AmB for injection.—The injection formulation of AmB is declared to contain 50 mg AmB in the glass bottle. The content of the bottle was transferred to a 25 mL volumetric flask and DMSO was added to the mark, yielding a concentration of 2.0 mg/mL. A 0.5 mL volume of this solution was transferred into a 100 mL volumetric flask, 0.5 mL methanol was added, and the solution was mixed. Subsequently, 5.0 mL of the piroxicam standard stock solution was added and the flask was filled to the mark with the mobile phase. The resulting working solution had an AmB concentration of 10 g/mL and a piroxicam concentration of 50 g/mL.

(c) AmB for tissue culture.—Tissue culture formulation of AmB is declared to have 50 mg AmB in a glass bottle. The content of the bottle was transferred to a 25 mL volumetric flask and DMSO was added to the mark, yielding a concentration of 2.0 mg/mL. A volume of 10.0 mL of the 2.0 mg/mL solution was transferred into a 20 mL volumetric flask and methanol was added to the mark, yielding a solution of AmB with a concentration of 1.0 mg/mL. Subsequently, 6.0 mL of this solution and 10.0 mL of the internal standard stock solution were transferred to a 100 mL volumetric flask, mixed, and the mobile phase was added to the mark, yielding an AmB concentration of 60 g/mL and a piroxicam concentration of 100 g/mL.

(d) AmB cream.—One-half gram AmB cream (equivalent to 15 mg AmB, as declared) was transferred to a 100 mL volumetric flask and water was added to the mark. The mixture was shaken until the solution was completely homogeneous (typically 30 min), yielding an aqueous solution with an AmB concentration of 150 g/mL. To remove lipid substances, 20 mL of this solution were extracted twice with 20 mL petroleum ether. One-half milliliter of this “lipid-free” aqueous solution (corresponding to 75 g AmB) was diluted with water to 10.0 mL and the entire volume was loaded onto a C18 Sep Pak cartridge. The cartridge was activated with 1 mL methanol and washed twice with 20 mL water before the sample was loaded. After the sample was loaded, the cartridge was washed with 5 mL acetonitrile and AmB was subsequently eluted with three 3.0 mL portions of 5% acetic acid in DMSO. The 3 eluates were combined. One milliliter internal standard solution (0.1 mg/mL piroxicam) was added to the combined solution prior to LC analysis.

(e) AmB lotion.—One-half milliliter lotion (equivalent to 15 mg AmB, as declared) was transferred to a 100 mL volumet-
Figure 2. (A) Liquid chromatogram of internal standard, piroxicam, and “bulk” AmB (Sigma): piroxicam concentration, 100 µg/mL, retention time, 3.97 min; bulk AmB concentration, 30 µg/mL, retention time, 12.41 min. (B) Liquid chromatogram of internal standard, piroxicam, and Fungizone for injection (AmB-FI): piroxicam concentration, 50 µg/mL, retention time, 4.03 min; AmB concentration, 10 µg/mL, retention time, 12.87 min. (C) Liquid chromatogram of internal standard, piroxicam, and Fungizone for tissue culture (AmB-FTC): piroxicam concentration, 100 µg/mL, retention time, 4.15 min; AmB concentration, 60 µg/mL, retention time, 11.45 min. (D) Liquid chromatogram of internal standard, piroxicam, and AmB cream (AmB-C), RS, USP: piroxicam concentration, 10.0 µg/mL, retention time, 4.51 min; AmB concentration, 7.5 µg/mL, retention time, 12.65 min. (E) Liquid chromatogram of internal standard, piroxicam, and AmB lotion (AmB-L), RS, USP: piroxicam concentration, 100 µg/mL, retention time, 3.95 min; AmB concentration, 50 µg/mL, retention time, 12.16 min.
Table 1. Results of LC and microbial assays of AmB

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC assay % detected</th>
<th>Microbial assay % detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk</td>
<td>94.1</td>
<td>91.0</td>
</tr>
<tr>
<td>Injection</td>
<td>102.0</td>
<td>102.4</td>
</tr>
<tr>
<td>Tissue culture</td>
<td>96.4</td>
<td>96.7</td>
</tr>
<tr>
<td>Cream</td>
<td>105.5</td>
<td>105.6</td>
</tr>
<tr>
<td>Lotion</td>
<td>97.6</td>
<td>106.1</td>
</tr>
</tbody>
</table>

a Comparison between the ratio of LC peak response of sample AmB with that of internal standard and the ratio of LC peak response of USP AmB RS standard with that of the internal standard.

b Comparison between the microbial growth inhibitory potency of sample with that of USP AmB RS standard.

Results and Discussion

Several mobile phases of different compositions were reported for the quantitation of AmB in biological samples: acetonitrile–10mM acetate buffer at pH 4.0 (37 + 63, v/v; 14), acetonitrile–10mM acetate buffer at pH 7.0 (40 + 60, v/v; 7), acetonitrile–acetic acid (10%)–water (41 + 43 + 16, v/v; 15), 2.5mM Na2EDTA–acetonitrile (70 + 30, v/v; 14, 17), or methanol–acetonitrile–2.5mM EDTA (50 + 35 + 15, v/v; 10). To achieve optimum separation between AmB and the internal standard, piroxicam, and to eliminate interferences from degradation products of AmB and, possibly, other components of the finished pharmaceutical preparations, we optimized the composition of the mobile phase. The amount of acetonitrile, water, and acetic acid in the mobile phase was systematically altered and the effect on retention time and separation of AmB and piroxicam was determined. An increase of acetonitrile from 40 to 50% shortened the retention times of both AmB and piroxicam; however, it caused interference with AmB and/or piroxicam degradation products. When the amount of acetonitrile was lowered to less than 40%, the retention time of AmB was significantly increased. When the amount of acetic acid was increased to more than 6% of the mobile phase, the stability of AmB decreased dramatically. On the other hand, acetic acid reduced to less than 6% of the mobile phase resulted in a broadened and asymmetrical peak for AmB. In addition to Bondapak column (300 3.9 mm id, C18, 10 m particle size), we also tested Ultrabase (250 4.6 mm id, C18, 5 m particle size), Ultrasphere (150 4.6 mm id, C18, 5 m particle size), and Perkin-Elmer (a cartridge column of 30 4.6 mm id, C18, 3 m particle size) columns. The retention time of AmB on Ultrabase or Ultrasphere columns was significantly longer than on a Bondapak column. This was obviously caused by the small packing particle size (5 m) of these 2 columns as compared with the Bondapak column’s 10 m particle size. On the other hand, the Perkin-Elmer column had a shorter retention time due to the shorter column length; however, this column did not provide acceptable resolution between AmB, piroxicam, and degradation products of AmB and/or piroxicam.

The isocratic elution (1.8 mL/min) of Bondapak C18 column (300 4.6 mm) with mobile phase consisting of acetonitrile–water–acetic acid (40 + 54 + 6, v/v) was found to be appropriate for the analysis of AmB. The injection volume was 40 L and the detector was set at 405 nm. A representative liquid chromatogram is presented in Figure 1. This LC method has an LOD of 10 ng/mL (3:1, signal-to-noise) and an LOQ of 30 ng/mL (10:1, signal-to-noise). The calibration curve for this method was linear from 0.01 to 100 g/mL (the equation of calibration curve is y = 63.6688x – 38.1129, where y is the area of AmB peak and x is the concentration of AmB in g/mL; correlation coefficient R² = 0.999148). AmB and the internal standard, piroxicam, had retention times of 12.4 and 4.0 min, respectively (Figure 1). The resolution between piroxicam and AmB was 10.6.

We used the developed LC method to determine the stability of AmB in stock and working solutions. Standard stock so-
olution of AmB in DMSO at 1.0 mg/mL was stable at 4°C and protected from light for 3 months. However, diluted solutions of AmB in DMSO are not very stable: at room temperature, 87% of AmB was lost in 8 days in a 0.1 g/mL solution. In addition, the working standard solutions of AmB obtained by dilution of standard stock solution with mobile phase (containing 6% acetic acid) should be prepared just prior to the analysis, because low pH promotes the degradation of AmB. For example, 64% of AmB was degraded in 4 h at room temperature when prepared as a 0.1 g/mL solution by diluting the DMSO stock solution with mobile phase. A standard working solution of AmB at 20 g/mL (prepared by diluting the DMSO stock solution with the mobile phase), stored overnight at room temperature in clear glassware without protection from regular laboratory light, suffered a 50% loss in AmB concentration. These results agree with previous studies which demonstrate that AmB is very sensitive to light, temperature, and pH values (18, 20).

The chromatograms of AmB analysis of Sigma’s AmB preparation (used here as “bulk”; Figure 2A), and AmB formulations for injection, tissue culture, cream, and lotion (Figures 2B–E) show good resolution between piroxicam and AmB. The chromatogram of cream formulation displays a large unidentified peak that elutes before piroxicam (Figure 2D). This peak probably corresponds to one of the inert ingredients of AmB cream formulation that is not eliminated during the cream sample cleanup process. However, this compound peak does not interfere with AmB analysis of cream formulation. The results of LC and microbial assays of AmB in bulk and formulated preparations of AmB are shown in Table 1. The USP RS standard AmB was used as a reference sample. The Sigma AmB displayed purities of 94.1 and 91.0% of AmB in comparison with the USP RS standard as determined by LC and microbial assay, respectively. The difference between the results of LC and microbial assays of the lotion formulation (Table 1) can be attributed to the fact that other components in the lotion may contribute to the inhibition of microbial growth and affect the results. However, according to USP, the AmB acceptable content range in the lotion formulation should not be less than 90.0% and not more than 125.0% of the labeled amount of AmB. Obviously, both the LC and microbial assays yielded results within the acceptable range.

Chromatograms of AmB formulations for tissue culture and cream (Figures 2C and D) displayed several peaks eluting after the internal standard, piroxicam. These peaks probably represent some excipients of formulations and are expected to be present in the chromatograms. Because the LC and microbial assays of the tissue culture and cream preparations correlated very closely (Table 1), we believe that these peaks have no effect on AmB quantitation by LC.

A simple and rapid LC method was developed for determination of AmB in bulk and formulated pharmaceuticals. In comparison with the official USP microbial assay for AmB, this LC method is more selective, sensitive, and offers positive identification.

Acknowledgments

We thank S.W. Zito for the early guidance during this research. We are grateful to the Northeast Regional Laboratory (NRL) and the Office of Regulatory Affairs (ORA) of the U.S. Food and Drug Administration (FDA) for facilitation of this study.

References