HPLC assay for determination of amphotericin B in biological samples


Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, Complutense University, 28040 Madrid, Spain
Lavetpa Veterinary Clinic, Benito Gutierrez 26, 28008 Madrid, Spain
Department of Parasitology, Faculty of Pharmacy, Complutense University, 28040 Madrid, Spain
Department of Animal Health, Faculty of Veterinary Medicine, Complutense University, 28040 Madrid, Spain

Received 19 June 2007; revised 13 September 2007; accepted 14 September 2007

ABSTRACT: A fast and selective HPLC method for assaying amphotericin B in biological samples was developed and validated. The chromatographic separation was achieved in less than 12 min on a reverse-phase C18 column using an acetonitrile–acetic acid–water (52:4.3:43.7, v/v/v) mixture as mobile phase. The flow rate was 1 mL/min and the effluent was monitored at 406 nm. A linear response over the concentration range 0.1–10.0 μg/mL was obtained. Intra-day and inter-day RSDs were below 5% for all the sample types. This new HPLC method was applied to assay amphotericin B in plasma and several tissue samples such as kidney, liver, spleen and bone marrow. Application of this method to pharmacokinetic studies in mice and dog is provided.

KEYWORDS: amphotericin B; high-pressure liquid chromatography; validation; pharmacokinetics

INTRODUCTION

The polyene antibiotic amphotericin B (AB), produced by Streptomyces nodosus, is one of the macrocyclic compounds containing both carboxyl and amino groups. AB has an amphipic character due to the rigid lipophilic chain of seven conjugated double bonds on one side of the macrocyclic ring and the hydroxyl groups on the opposite side, and this characteristic structure is believed to be important in its biological action.

AB remains the reference treatment for invasive fungal infections such as candidiasis, coccidiosis, criptococosis, histoplasmosis and aspergillosis (Barrett et al., 2003; Gallis et al., 1990), as well as for the treatment of leishmaniasis (Barrat et al., 2005; Murray, 2004; Olívar et al., 2005). However, the use of AB as mixed-micellar dispersion with sodium deoxycholate (Fungizone®, Bristol-Myers Squibb, USA) is limited by toxicity, with both infusion-related effects and cumulative nephrotoxicity. To reduce this toxicity, several lipid-based formulations of AB have been developed, such as a liposomal formulation (AmBisome®, Nextra Pharmaceuticals, USA), a lipid complex of AB with phospholipids (Abelcet®, Liposome Company Inc., USA) and a colloidal dispersion of AB with cholesteryl sulfate (Amphocil®, Sequus Pharmaceuticals Inc., USA), and these are now available in several countries (Tiphine et al., 1999). Although each of these novel products has its own pharmaceutical composition and pharmacokinetics, there is still only limited data about their optimal therapeutic dosages and disposition kinetics, so it is very important to be able to control levels in plasma and all media where new AB formulations (like liposomes, complexes, nanoparticles or colloidal pastes) are assayed. Therefore, there has been much interest in developing fast and reliable measurements of AB plasma, whole blood and tissue concentrations using validated assays. With this aim, different methods have been described in order to analyze biological samples containing AB. For instance, there are complex HPLC methods that can separate free AB, protein-bound AB and lipid-formulated AB in plasma (Egger et al., 2001; Bekersky et al., 2002). Although some pharmacopoeias recommend the use of microbiological assays for quantitative determination of AB (The United States Pharmacopeia, 2006), HPLC methods have been demonstrated to be easier, faster, and more accurate and reproducible.

The purpose of the present work was to develop a fast and reliable HPLC method for quantitation of AB in different biological samples that could be performed...
easily in research and routine clinical laboratories with currently available equipment and materials. Most of HPLC methods for AB quantitation reported in the literature used salts in their mobile phase (Alakh et al., 1996; Eldem and Arican-Cellat, 2001; Egger et al., 2001; Hosotsubo and Hosotsubo, 1989). Salts significantly increase the risk of saturation, breakdown or overpressure in the column and reduce the life-time of the column. Although some other HPLC methods without salts in their mobile phase have been reported (Echevarría et al., 1998; Lue et al., 2002), in our experimental conditions with those methods it was not possible to obtain a correct separation for AB. Therefore, a new HPLC method for monitoring AB in aqueous/methanol mediums suitable for assay of biological samples has been developed and is described and validated.

EXPERIMENTAL

Materials

AB raw material was a gift from Squibb Bristol Myers, Barcelona, Spain. HPLC-grade acetonitrile and methanol (MeOH) were purchased from Labsciences, Dublin, Ireland. Acetic acid was supplied by Panreac S.A., Barcelona, Spain. Sodium hydroxide was provided by Panreac S.A., Barcelona, Spain. Deionized water was obtained from a Milli-Q water purification system (Millipore, USA).

Apparatus and chromatographic conditions

High-performance liquid chromatography was performed with a modular liquid chromatograph equipped with a Jasco PU-1580 pump, a Gilson 231 XL autosampler fitted to a Jasco UV-1575 UV–visible detector. Integration of the peaks was performed with the program Borwin 1.5 for PC (JMBS Developments). Compounds were separated on a 250 × 4.6 mm, 5 μm particle size Thermo Hypersil BDS C18 reverse-phase column.

Elution was carried out isocratically with a mobile phase consisted of an acetonitrile–acetic acid–water (52:4.3:43.7, v/v/v) mixture filtered through a 0.45 μm hydrophilic polypropylene filter membrane (GH polyprop, Pall Corp., USA) and degassed. It flowed at a constant flow rate of 1 mL/min and the effluent was monitored at 406 nm. The total chromatographic run time was 15 min. Under these conditions, the relative retention time of AB was approximately 12 min.

Standard solutions

Stock solutions were prepared by accurately weighing 15 mg of AB in a 100 mL volumetric flask, dissolved and diluted to volume with pH 11 sodium hydroxide aqueous medium, to obtain a concentration of 150 μg/mL. Stock solutions were further diluted with an MeOH:H2O (2:1, v/v) mixture to obtain the following calibration standards: 0.1, 0.3, 1, 3 and 10 μg/mL; all these calibration standards, obtained from each stock solution, were analyzed in triplicate.

Validation of the assay

Linearity. The linearity was evaluated with diluted stock standard solutions containing 0.1, 0.3, 1, 3 and 10 μg/mL of AB. For each concentration three measurements were performed and calibration lines were constructed. The data were statistically evaluated using a regression analysis software package (Statgraphics Plus 5.0, Manugistics, USA) and the equations were adjusted by means of least-square linear regression analysis. The slope, intercept and determination coefficients of each calibration line together with the mean and standard deviation (SD) were determined.

Accuracy. The recoveries of AB were determined by spiking an amount of the drug into blank MeOH:H2O (2:1, v/v), plasma and tissue (liver, spleen and kidney) samples respectively. Recoveries were evaluated by analyzing samples at the following concentrations: 0.1, 3 and 10 μg/mL.

Plasma samples were prepared by mixing four parts of blank plasma, obtained from human volunteers, and one part of MeOH:H2O (2:1 v/v) samples containing different known concentrations of AB. In this way, AB plasma:MeOH (4:1 v/v) samples of 0.1, 3 and 10 μg/mL were obtained and then they were deproteinized with 2 vols of MeOH. All these samples were vortexed for 1 min and centrifuged at 4500 rpm for 10 min. Supernatant was filtered through a 0.45 μm sterile syringe filter (Milllex®, Millipore Millex HV-1) and injected into the HPLC.

Tissue samples were obtained from mice. Mice were sacrificed by chloroform inhalation, and the liver, spleen and kidneys were sonicated in water, and four parts of MeOH:H2O (2:1) containing different known amounts of AB, in order to obtain AB concentrations of 0.1, 3 and 10 μg/mL, were added to one part of the homogenate. The resulting mixtures obtained from liver, spleen and kidneys were vortexed for 1 min and centrifuged at 4500 rpm for 10 min. Supernatants were filtered through a 0.45 μm sterile syringe filter (Milllex®, Millipore Millex HV-1) and injected into the HPLC.

The individual and the overall mean percentage recovery were calculated and a relative standard deviation (RSD) value of maximum 5% was set as the acceptance criterion.

Intraday and inter-day precision. Intraday precision was defined as RSD for the peak areas calculated from three samples at concentrations of 0.1, 3 and 10 μg/mL, respectively, on the same day (n = 3).

Inter-day precision was calculated using the values measured from nine different samples (three samples from each different day) at the concentrations of 0.1, 0.3, 1, 3 and 10 μg/mL, respectively. The coefficient of variation for the peak areas was calculated.

Sensitivity. The sensitivity in terms of detection and quantitation limits was determined based on signal-to-noise ratio, by comparing measuring signals from samples with 0.1 μg/mL of AB with those of blank samples. Signal-to-noise ratios of 3 and 10 were considered acceptable for estimating the detection limit and quantitation limit respectively (ICH, 1996).

Selectivity. The selectivity of the HPLC method was checked by analyzing different independent blank human
plasma samples. The chromatograms of the blank plasma samples were compared with the chromatograms obtained by plasma spiked with AB.

**Application of the method to pharmacokinetic and tissue distribution studies**

As an example, the proposed HPLC method was applied to determine the plasma concentration–time profiles of AB following intravenous bolus administration of a new AB formulation, consisting of albumin microspheres containing AB (AB-Mic) elaborated by a spray-drying process (Sánchez-Brunete et al., 2004), in dog and mice. AB-Mic was prepared by dispersing of AMB in 5 mL of a water solution formed by sodium deoxycholate, dibasic sodium phosphate and monobasic sodium phosphate. The resulting dispersion was subjected to moderate stirring to achieve a homogeneous suspension. A 20% serum albumin solution (5 mL) was added, and the final mixture was spray-dried using a Büchi B 191 spray-drier to obtain albumin microspheres containing AMB.

Female albino ICR mice (25–30 g) were housed in groups of six in plastic cages in a 12 h dark–light cycle animal facility with controlled temperature (25 °C) and humidity (70%); water and food were unrestricted throughout the study. The preparation was administered by intracardiac injection at dose of 2 mg of AB per kg (body weight). Immediately before intracardiac administration, the preparation was reconstituted with sterile 5% dextrose in water. AB concentrations in serum and organs were determined using high-pressure liquid chromatography. Blood samples were obtained by the retroorbital route at 5 and 90 min, 6, 18 and 40 h after drug injection; plasma was separated by centrifugation (10,000 rpm for 10 min) and AB was extracted from plasma in MeOH. Mice were then sacrificed by chloroform inhalation, and the liver, spleen and kidneys were removed and homogenized with MeOH. Three mice were used for each time point.

A single dose of 5 mg of AB per kg (body weight) was administered intravenously in a dog. Blood samples from the dog were taken at 15, 30, 50, 110 and 1380 min after the administration, and collected in heparinized tubes. The plasma was separated by centrifugation (10,000 rpm for 10 min). Also, a bone marrow sample was taken at the end of the study. All biological specimens were stored at −20 °C until analysis; under these conditions AB was found to be stable in these biological matrices.

**RESULTS AND DISCUSSION**

**Validation**

Chromatograms of blank plasma and AB-spiked aqueous and plasma samples are shown in Fig. 1. In all the cases, AB signal was clearly distinguished with great resolution, and when assaying plasma no interfering peak at the retention time for AB was present in blank plasma or plasma samples spiked with AB.

Quantification based on peak-area of AB was found to yield more consistent and reproducible results than peak-height calculations. Linear regression analysis of the dependence of peak area response (y) on the theoretical concentration (x) gave the equation $y = -27,040.7 + 348,726x$. The correlation coefficient was $r^2 = 0.997$. This coefficient confirmed the linearity of the method over the concentration range assayed (0.1–10 μg/mL).

Table 1 shows the ANOVA analysis of the linearity study. The $R$-squared statistic indicates that the model as fitted explains 99.7% of the variability in AUC. Since the $p$-value in the ANOVA table was less than 0.01, there was a statistically significant relationship
HPLC assay for determination of amphotericin B in biological samples

Table 1. Analysis of variance with the lack-of-fit test

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>d.f.*</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>$7.6213 \times 10^{13}$</td>
<td>1</td>
<td>$7.6213 \times 10^{13}$</td>
<td>15,057.42</td>
<td>0.0000</td>
</tr>
<tr>
<td>Residual</td>
<td>$2.1764 \times 10^{11}$</td>
<td>43</td>
<td>$5.0615 \times 10^{9}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack-of-Fit</td>
<td>$9.1939 \times 10^{10}$</td>
<td>13</td>
<td>$7.0722 \times 10^{9}$</td>
<td>1.69</td>
<td>0.1158</td>
</tr>
<tr>
<td>Pure Error</td>
<td>$1.2570 \times 10^{11}$</td>
<td>30</td>
<td>$4.1901 \times 10^{9}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>$7.4630 \times 10^{13}$</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Degrees of freedom.

Table 2. Intra-day variabilities of the HPLC assay for AB

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Concentration (µg/mL) found (mean ± SD; n = 3)</th>
<th>Found (%) a</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.105 ± 0.001</td>
<td>104.7 ± 1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>3.047 ± 0.052</td>
<td>101.5 ± 1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>10</td>
<td>9.859 ± 0.288</td>
<td>98.6 ± 2.9</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* a Calculated from concentration and expressed as recovery in percent, mean ± SD, n = 3.

Table 3. Inter-day variabilities of the HPLC assay for AB

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Found (%) b</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.109 ± 0.003</td>
<td>0.107 ± 0.006</td>
<td>0.109 ± 0.004</td>
<td>108.8 ± 1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>0.3</td>
<td>0.315 ± 0.011</td>
<td>0.324 ± 0.004</td>
<td>0.329 ± 0.014</td>
<td>107.7 ± 2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>1</td>
<td>0.982 ± 0.059</td>
<td>0.981 ± 0.068</td>
<td>0.977 ± 0.015</td>
<td>98.0 ± 0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>3.047 ± 0.052</td>
<td>2.832 ± 0.070</td>
<td>2.892 ± 0.044</td>
<td>97.5 ± 3.6</td>
<td>3.7</td>
</tr>
<tr>
<td>10</td>
<td>9.859 ± 0.288</td>
<td>9.705 ± 0.348</td>
<td>10.517 ± 0.057</td>
<td>100.3 ± 4.4</td>
<td>4.4</td>
</tr>
</tbody>
</table>

* a Mean ± SD, n = 3.

Table 4. Accuracy of the method for analysis of AB in spiked MeOH:H2O samples

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>99.389 ± 1.293</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>100.567 ± 2.214</td>
<td>2.2</td>
</tr>
<tr>
<td>10</td>
<td>99.736 ± 1.065</td>
<td>1.1</td>
</tr>
<tr>
<td>Mean ± SD, n = 15</td>
<td>99.897 ± 1.403</td>
<td>1.4</td>
</tr>
<tr>
<td>95% confidence limits</td>
<td>101.041 &gt; µ &gt; 98.753</td>
<td></td>
</tr>
</tbody>
</table>

* a Expressed as recovery in percent, mean ± SD, n = 3.

Table 5. Accuracy of the method for analysis of AB in spiked plasma samples

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>104.049 ± 0.884</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>103.958 ± 1.028</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>103.196 ± 1.963</td>
<td>1.9</td>
</tr>
<tr>
<td>Mean ± SD, n = 9</td>
<td>103.734 ± 1.188</td>
<td>1.1</td>
</tr>
<tr>
<td>95% confidence limits</td>
<td>177.564 &gt; µ &gt; 97.325</td>
<td></td>
</tr>
</tbody>
</table>

* a Expressed as recovery in percent, mean ± SD, n = 3.

between AUC and concentration at the 99% confidence level. Furthermore, since the p-value for the lack-of-fit test in the ANOVA table was greater than 0.10, the lineal model appeared to be adequate for the observed data.

The detection and quantification limits of the assay for AB were found to be 0.016 and 0.054 µg/mL, respectively.

The intraday variabilities of the assay method are shown in Table 2 and the corresponding inter-day variabilities in Table 3. These data indicated that the assay method was reproducible within the same day and within different days; RSDs were less than 5% for the all samples over the concentration range assayed.

The accuracy of the assay method in determining AB concentrations in MeOH:H2O and spiked plasma specimens, respectively, is presented in Tables 4 and 5. The deviation from theoretical values was below 5% at all concentration levels studied for each sample type. For tissue samples the values of accuracy obtained were similar to the plasma specimens, and the deviation from theoretical values was also below 5% at all concentration levels for liver, spleen and kidney samples.
Application of the method to pharmacokinetic study

The pharmacokinetics of AB in plasma, liver, spleen and kidney in mice is shown in Fig. 2. AB concentrations decreased faster in plasma than in the other biological tissues. Figure 2 clearly shows how AB was specially accumulated in spleen and liver, whereas AB levels in kidney were relatively low. Similar AB tissue distribution have been previously reported with AB lipid formulations (Andes et al., 2006; Olson et al., 2006).

AB is a useful drug for the treatment of canine visceral leishmaniasis (Lamothe, 2001; Noli and Auxilia, 2005). This disease is caused by the parasite *Leishmania infantum*. This parasite is accumulated in the reticulo-endothelial system and for this reason high AB concentrations in liver, spleen and bone marrow are required to eradicate the parasite. AB is a drug with a narrow therapeutic index and so drug monitoring is recommended at least during the first drug development stages with a new formulation. For this reason a preliminary limited pharmacokinetic study has been performed in a dog.

Figure 3 shows the plasma AB concentrations in dog after an intravenous administration of 5 mg/kg of the AB-Mic formulation. Specially relevant is the possible AB accumulation in bone marrow where the parasite is usually present. In our experimental conditions, the dog bone marrow AB concentration at 24 h was five times higher than that of plasma.

CONCLUSIONS

It can be concluded from the present work that the proposed HPLC method was useful to detect with high linearity, sensitivity and precision AB in aqueous samples, which was useful for measuring with substantial sensitivity and reliability the drug in different conditions. According to this method, there was no need to employ salts, and therefore the risk of saturation, breakdown or overpressure in the column was reduced. Also, the method has been used to determine plasma concentrations and it has been successfully applied to pharmacokinetic study of this drug in mice and dog after a single administration of AB-Mic, a new formulation of AB. In this way, the method described was sensitive enough for the quantitative determination of AB in different biological tissues. Moreover, the simplicity and efficacy of the developed method as well as the relatively short retention times of AB allowed the analysis of a large number of samples in a short time, providing a fast and inexpensive method for the therapeutic AB monitoring in clinical laboratories.

Acknowledgments

We thank Squibb Bristol Myers for having supplied us with amphotericin B. This work was partially supported...
HPLC assay for determination of amphotericin B in biological samples by a grant from the Complutense University and Madrid Community Administration to the research group 910939 and from Science and Technology Inteministerial Commission, Government of Spain (project AGL2002-02175 GAN).

REFERENCES


