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Determination of Amphotericin B in cerebrospinal fluid by solid-phase extraction and liquid chromatography

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Abstract

A highly sensitive and reproducible liquid chromatography (LC) method for the determination of Amphotericin B in cerebrospinal fluid has been developed and validated. This LC-based method involves using nystatin as an internal standard and solid-phase extraction for sample preparation, followed by reversed-phase separation monitored by absorbance at 410 nm. The method has a limit of quantification of less than 1 ng ml^{-1} and excellent precision and accuracy, with both percentage relative standard deviation and percentage relative error less than 10%. The established linearity range was 1–10 ng ml^{-1} ($r^2 > 0.99$). The extraction recovery of Amphotericin B from the cerebrospinal fluid is higher than 90% over the entire linear range. The method has been successfully employed for studying the penetration of Amphotericin B into the central nervous system in dogs and human.

Keywords: Amphotericin B; Analysis; Cerebrospinal fluid; Internal standard; LC; Solid-phase extraction

1. Introduction

Amphotericin B is commonly used for the treatment of systemic mycoses. However, its effectiveness for treating infections in the central nervous system (CNS) has been greatly limited by its poor penetration through the blood–brain barrier (BBB) [1–3] and its nephrotoxicity [4–7]. Recently, a novel drug delivery system called RMP-7 [8,9], which is capable of permeabilizing the BBB, has been investigated for its capability of facilitating the entry of small molecules into the CNS. In order to study the potential utility of the drug delivery system for Amphotericin B, it is necessary to determine concentrations of Amphotericin B in cerebrospinal fluid (CSF) in animals or man. The measurement requires a highly sensitive and specific analytical method because of the low concentration of Amphotericin B and the limited sample volume of CSF.

Several LC-based methods for quantifying Amphotericin B in plasma, serum and urine have been published in the literature [10–17]. However, these methods typically have a limit of quantification above 5 ng ml^{-1} and lack the sensitivity required for this particular application. In this report, we describe a sensitive and reproducible analytical method for the determination of Amphotericin B in cerebrospinal fluid with a limit of quantification in the range of less than 1.0 ng ml^{-1}. The LC-based method involves using nystatin as internal standard and solid-phase extraction for sample preparation. The method was validated for precision, accuracy, linearity, and stability of Amphotericin B under various conditions. It has been successfully applied to the analysis of Amphotericin B in both animal and human CSF samples at low ng ml^{-1} levels.

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2. Experimental

2.1. Chemicals and materials

USP grade Amphotericin B was obtained from Amresco (Solo, OH). Reagent grade nystatin was purchased from Sigma (St. Louis, MO). Blank pooled dog cerebrospinal fluid was obtained from North Biomedical Research (Muskegon, MI). BakerBond C18 spe cartridges were obtained from J.T. Baker (Phillipsburg, NJ). Other chemicals, reagents and materials included methanol (LC grade), acetonitrile (LC grade), disodium ethylenediamine (reagent grade), sodium bicarbonate (reagent grade) and dimethylsulfoxide (DMSO, LC grade).

2.2. Preparation of standard solution and control samples

Stock solutions of Amphotericin B and nystatin (internal standard, IS) were prepared fresh every six months by dissolving a known weight of each in a mixture of DMSO and methanol (1:1, v/v). Serial dilutions of the stock solutions were made using methanol. Standards of Amphotericin B were freshly prepared by spiking known amounts of the Amphotericin B (100 μl) working stock solutions into 10 ml of a mixture of water/methanol (9:1, v/v). Quality control samples were prepared by spiking known amounts of the Amphotericin B working stock solutions into 50 ml of the blank dog CSF. Aliquots (1.1 ml) of the quality control samples were pipetted into a series of cryotubes for storage at −80°C. The concentration of Amphotericin B in standard solutions ranged from 1.0 to 10 ng ml^{-1}. Quality control samples were prepared at concentrations of 1.5, 3.5 and 7 ng ml^{-1}.

2.3. Extraction procedures

Unknown CSF sample, control or standard (1.0 ml) was placed in a 75 × 12 mm glass tube. After addition of 50 μl of a 10 μg ml^{-1} solution of internal standard, the tube was vortexed briefly. The sample was applied to a BakerBond C18 SPE cartridge which was previously conditioned with 3 ml of methanol and 3 ml of carbonate buffer (pH 9; 0.1 M). The cartridge was washed with 2 ml of the carbonate buffer and was allowed to air dry for ≈2 min. The cartridge was then eluted with two 0.5 ml aliquots of methanol. The eluent was collected and was dried under a stream of nitrogen. The residues were reconstituted in 200 μl of methanol and transferred into LC vials for analysis.

2.4. LC analysis

The chromatographic system employed was an automated Hewlett-Packard 1050 system with a ChemStation data system (version B.02.04). The analytical column, Nova-Pak C18 (4 μm particle size, 150 × 3.9 mm) was obtained from Waters Associates (Milford, MA). The mobile phase was a mixture of 0.01 M EDTA (pH 5)/acetonitrile (65:35, v/v) and the flow rate was 0.5 ml min^{-1}. An injection volume of 100 μl was used. The wavelength of the detector was set at 410 nm. The run time was 10 min.

Standard curves were obtained by plotting the peak height ratio (peak height of Amphotericin B/peak height of internal standard) versus the concentration of Amphotericin B. The concentration of Amphotericin B in CSF samples was determined by interpolation from the linear regression curve.

3. Results and discussion

For the determination of Amphotericin B in biological fluids using LC, both protein precipitation [10–13] and solid-phase extraction [14–17] have been used for sample preparation. Solid-phase extraction was reported to give cleaner extracts and, consequently, higher sensitivity than simple protein precipitation [15,16]. A number of compounds, including *p*-nitrobenzyyloxyamine [13], 1-amino-4-nitronaphthalene [16] and disperse yellow 42 dye [14], have been used as internal standards when protein precipitation was employed for sample clean-up. These compounds, however, were found unsuitable as internal standard when solid-phase extraction was utilized [17]. One paper reported that *N*-acetyl-Amphotericin B, which is not readily available commercially, can be used as internal standard with solid-phase extraction [16]. In this study, nystatin, which has a very similar structure to Amphotericin B, was used as interal standard.

Figs. 1(a) and 1(b) are typical chromatograms of extracted blank dog CSF and blank dog CSF spiked with internal standard.

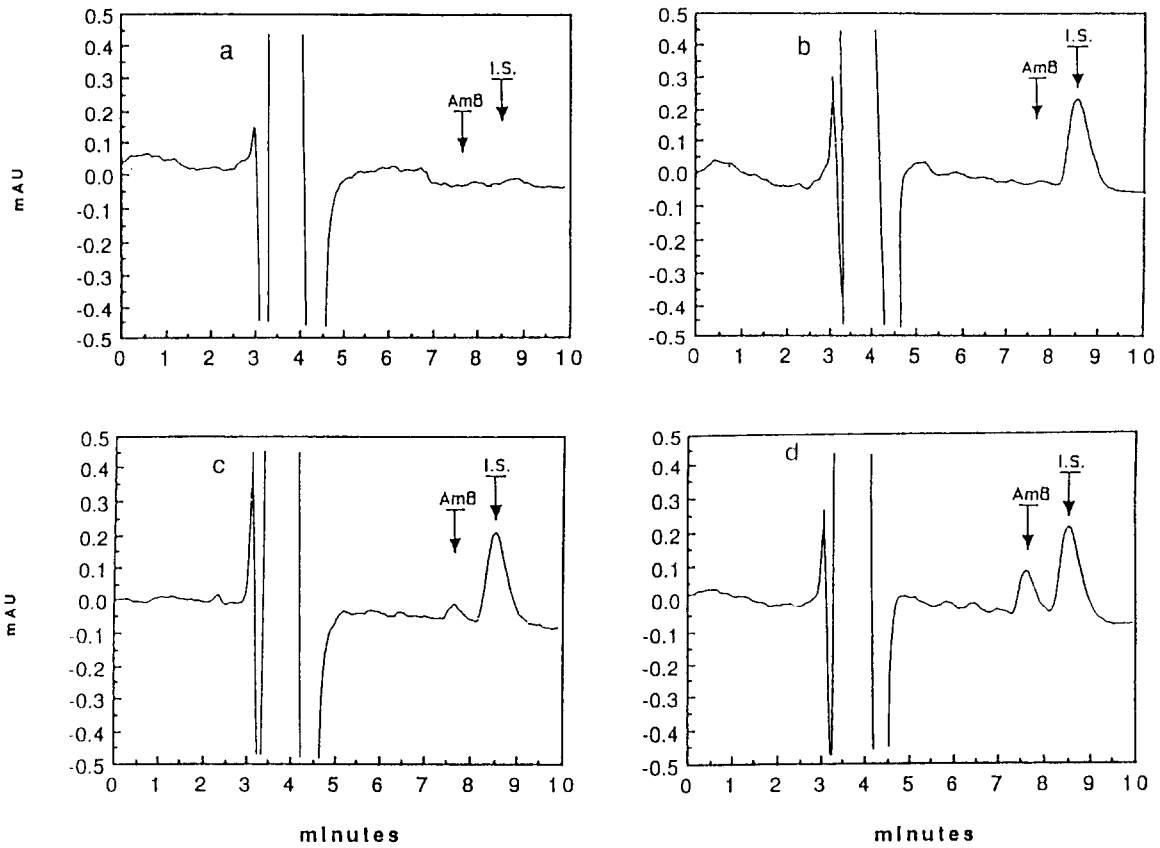

Fig. 1. Chromatograms of extracted (a) blank CSF, (b) blank CSF spiked with internal standard, (c) blank CSF spiked with internal standard and 1.0 ng ml^{-1} of Amphotericin B, and (d) CSF sample obtained from a dog 2 h after it was dosed with AmB.

Fig. 1(c) is a chromatogram of extracted blank dog CSF spiked with internal standard and 1.0 ng ml^{-1} of Amphotericin B. Fig. 1(d) shows a chromatogram of an extracted CSF sample collected from a dog 2 h after it was dosed intravenously with Amphotericin B (the calculated concentration of Amphotericin B in the sample is 2.5 ng ml^{-1}). The extracts of the blank CSF give a relatively clean background and have no significant interfering peaks for either Amphotericin B or internal standard. Amphotericin B and the internal standard are well resolved and their retention times are 7.6 and 8.5 min, respectively. Based on a signal-to-noise ratio of 3, the limit of detection is below 0.5 ng ml^{-1}.

3.1. Extraction recovery

The purpose of the SPE procedure was not only to remove the interfering components, but also to concentrate Amphotericin B (concentration factor = 5) in the sample prior to LC analysis to improve the sensitivity of the method. The extraction recovery of Amphotericin B from a CSF sample at a particular concentration was determined by comparing the peak height of its extracts with that of an Amphotericin B standard prepared in methanol at a concentration five times higher. The recoveries of Amphotericin B from both the standards and the CSF samples over the concentration range of 1–10 ng ml^{-1} are more than 90%. Owing to the fact that nystatin has a much lower specific absorptivity than Amphotericin B at 410 nm, a concentration of 500 ng ml^{-1} of nystatin in standards and CSF samples was used for internal standard in order to obtain a chromatographic peak whose size was comparable to that of Amphotericin B over the applicable concentration range. Experiments showed that the extraction recovery of the internal standard is higher than 95%.

3.2. Linearity

Five Amphotericin B standards covering the concentration range from 1 to 10 ng ml^{-1} were

Table 1
Precision and accuracy of analytical runs

	Concentration (ng ml^{-1})			
	Added	Measured	RSD (%)[a]	Relative error (%)
Run #1				
	1.5	1.36	4.4	−9.4
	3.5	3.45	2.9	−1.4
	7.0	7.61	4.6	+8.7
Run #2				
	1.5	1.42	6.3	−5.3
	3.5	3.44	7.5	−1.7
	7.0	6.35	1.7	−9.3
Run #3				
	1.5	1.48	6.6	−1.7
	3.5	3.58	8.4	+2.3
	7.0	6.61	4.5	−5.6

[a] $n = 6$.

analyzed on several different days. The peak height ratio of Amphotericin B and internal standard versus concentration were fitted into a linear function using non-weighted least squares regression analysis. Four standard curves were run on different days over a period of two weeks. The values of their slopes are 0.1318, 0.1558, 0.1462 and 0.1698, and those of their intercepts are 0.01068, −0.01985, −0.02750 and 0.02905. The values of the y-intercept for the four standard curves are not statistically different from zero at the 95% confidence level. The standard curves showed excellent linearity over the concentration range studied. With less than 10% deviations, the back calculated values agreed with the theoretical values of the standards. The correlation coefficients (r^2) of the standard curves were all larger than 0.99.

3.3. Precision and accuracy

Six quality control samples at each of the three concentrations (1,5, 3.5 and 7 ng ml^{-1}) were analyzed on three separate runs over a period of 2 weeks. The results are presented in Table 1. The method showed excellent precision and accuracy with the relative standard deviation (RSD) ranging from 1.7% to 8.4% and the relative error ranging from −9.4% to +8.7%.

3.4. Stability

The stability of Amphotericin B during sample processing (in CSF at room temperature and freeze/thaw cycles), chromatographic analysis (in methanol at room temperature), and sample storage at −80°C were investigated with the quality control sample of 3.5 ng ml^{-1}. A series of 1.0 ml aliquots of a freshly spiked CSF sample were allowed to set at room temperature for various periods of time prior to analysis. Fig. 2 shows the percentage of Amphotericin B remaining in the CSF sample after various time periods. The loss of Amphotericin

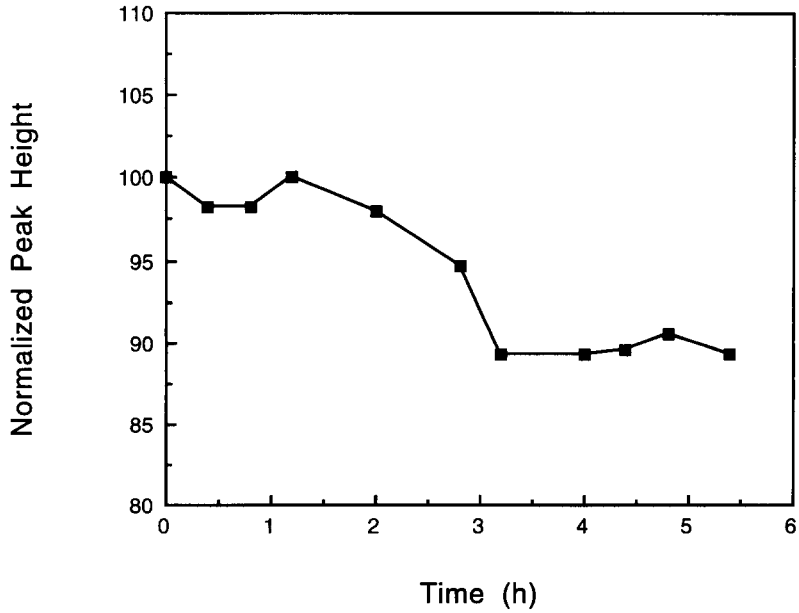

Fig. 2. Stability of Amphotericin B in CSF matrix at room temperature.

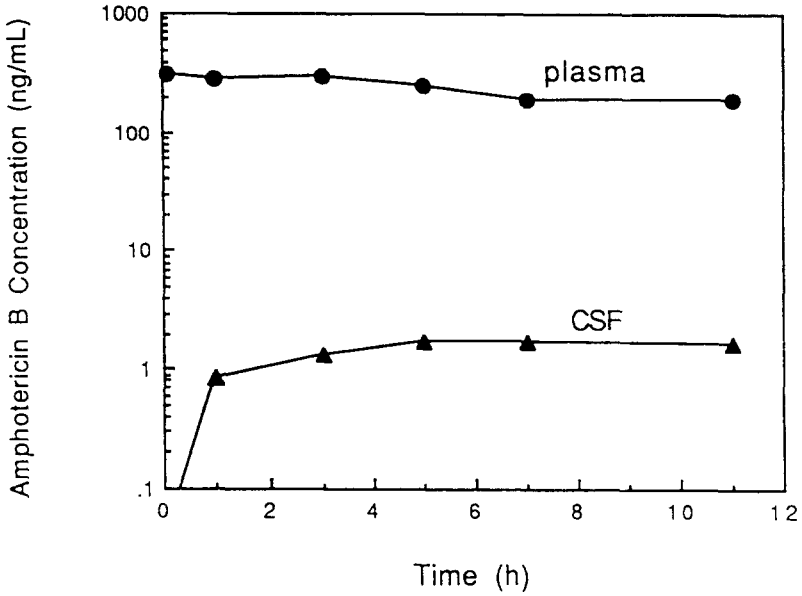

Fig. 3. Concentrations of Amphotericin B in plasma and CSF of a dog at various time points after it received an intravenous dose of Amphotericin B.

B is minimal during the first 2 h and is ≈10% during the next 3 h. The loss of Amphotericin B in CSF after two freeze/thaw cycles is not significant (<5%). Amphotericin B in the processed solution (i.e. methanol) is stable (loss < 5%) at room temperature for at least 10 h.

4. Conclusion

The method has been successfully applied to the determination of Amphotericin B concentrations in dog and human (data not shown) CSF samples after intravenous administration of Amphotericin B. Fig. 3 shows the CSF and plasma concentration–time curves of a single dog following an intravenous infusion of 0.3 mg kg^{-1} of Amphotericin B. Amphotericin B in the plasma samples was measured according to the method of Granich et al. [16]. The CSF concentration of Amphotericin B is very low (< 5 ng ml^{-1}), especially during the initial time period following dose. The CSF/plasma concentration ratio of Amphotericin B is a quantitative measure of the penetration of Amphotericin B into the CNS. The CSF concentration is generally less than 1% of that in the plasma.

In conclusion, a sensitive, specific, accurate and reproducible LC method has been developed. The high sensitivity of the method made it possible to quantitatively study the penetration of Amphotericin B into the CNS in animal models and in humans.

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