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Short communication

Amphotericin B determination in respiratory secretions by reversed-phase liquid chromatography

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Abstract

Direct delivery of amphotericin B (AMB) to the respiratory tract may be an alternative to intravenous administration. The use of inhalation allows high AMB concentrations to be achieved at the site of infection. A reversed-phase high-performance liquid chromatographic method with a 30-mm-long column is described for assaying AMB in respiratory secretions obtained by bronchoaspiration (BAS) and bronchoalveolar lavage (BAL). Sample clean-up involved treatment with methanol (BAS) and solid-phase extraction onto Sep-Pak C_{18} cartridges (BAL). The mobile phase consisted of 2.5 m*M* Na_2EDTA–acetonitrile (70:30, v/v). The retention time of AMB was 1.5 min. The range of the assay was from 0.1 to 5 μg/ml. The mean recovery was over 90% for both fluids. Within-day and between-day RSDs ranged from 3.10 to 11.87%. AMB in the BAS samples was stable for two days at 20–25°C, fifteen days at 4°C and for three months at −20°C. The drug in the BAL fluid was stable for one day at 20–25°C, seven days at 4°C and for one month at −20°C. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Amphotericin B (AMB) is a polyene antibiotic produced by the fungus *Streptomyces nodosus*. AMB binds to ergosterol, the sterol present in cell membranes, and destroys the membranes [1]. After more than 30 years of clinical use, AMB remains the drug of choice for the treatment of serious systemic fungal infections [2,3]. Invasive pulmonary aspergillosis (IPA) is a fungal infection associated with high morbidity and mortality rates in neutropenic and immunosuppressed patients [2–4].

The usefulness of intravenous AMB has been limited by toxicity [2,3], and direct delivery of AMB to the respiratory tract of patients with IPA may be an alternative to intravenous administration. The use of inhalation allows high AMB concentrations to be achieved at the site of infection, with a reduced risk of systemic toxic reactions [4,5]. Some studies have reported its efficacy and safety in the prophylaxis of IPA in neutropenic patients subjected to bone marrow transplantation [6–8]. The major advantage of the aerosolized administration of AMB is the opportunity for self-administration without the need for hospitalization.

The methods for measuring AMB by HPLC reported in the literature have been developed for several biological fluids (serum, urine) and tissues [9–12], but not for respiratory samples. The pharmacokinetics of this drug in human respiratory secretions administered by aqueous inhalation is presently unknown.

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This paper describes a modification of an isocratic reversed-phase HPLC method in a short column, reported by us for use in serum [11], for the determination of AMB respiratory secretions obtained by bronchoaspiration (BAS) and bronchoalveolar lavage (BAL). The procedure was evaluated in a clinical setting to determine its usefulness in investigating the pulmonary disposition of the drug in patients receiving aerosolized AMB.

2. Experimental

2.1. Standards and reagents

Sodium desoxycholate of AMB (Fungizone) was kindly supplied by Squibb (Princeton, NJ, USA). Ethylenediaminetetraacetic acid disodium dihydrate ($Na_2EDTA \cdot 2H_2O$) and sodium acetate trihydrate ($Na_2C_2H_3O_2 \cdot 3H_2O$) were obtained from Sigma (St. Louis, MO, USA), methanol and acetonitrile from Merck (Darmstadt, Germany) and the Sep-Pak C_{18} cartridges [Vac 3cc (500 mg) for solid-phase extraction] were from Waters (Milford, MA, USA). All chromatographic solvents were of HPLC grade, and all other chemicals were of analytical grade.

2.2. Instrumentation

Analyses were performed on a Kontron chromatograph (Milan, Italy) equipped with a Model 325 solvent-delivery system, a Model 465 automated sample injector with variable injection volume, and a Model 432 ultraviolet absorption variable-wavelength detector with an 8-μl flow cell. The detector response was monitored by an Acer 1120 SX computer with Kontron PC-integrator software, version 3.00.

2.3. Preparation of standard solutions

Aqueous stock solutions containing 5 mg/ml of AMB were prepared and stored at −80°C. Working standard solutions were prepared as needed by diluting the stock solutions with methanol to yield final concentrations of 0.1, 0.25, 0.5, 1, 2.5 and 5 μg/ml.

2.4. Extraction procedure

2.4.1. BAS

The total volume of BAS was mixed with methanol (1:1, v/v), homogenized by vortex-mixing for 1 min and centrifuged for 20 min at 3000 rpm.

2.4.2. BAL

The first part of sample preparation was similar to that used for BAS. The volume of BAL fluid was quantified and was mixed with methanol (1:1, v/v). After centrifugation for 20 min at 3000 rpm, the supernatant was combined with 0.01 *M* sodium acetate buffer, pH 7.4 (v/v). The mixture was transferred to a Sep-Pak C_{18} cartridge (Waters) that had been conditioned previously with acetonitrile (3 ml×2), followed by sodium acetate buffer (3 ml×2). After the sample was loaded, the column was flushed with 3 ml of methanol–sodium acetate buffer (1:1, v/v), five times. AMB was eluted with methanol (1.5 ml×2). The final eluate (3 ml) was evaporated in a nitrogen atmosphere to dryness. The dried extract was reconstituted in 400 μl of methanol and vortex-mixed for 15 s.

2.5. Chromatographic conditions

Separation was done with a Perkin-Elmer ODS column, 3 μm, 30×4.6 mm I.D. (Norwalk, CT, USA). The mobile phase consisted of 2.5 m*M* Na_2EDTA–acetonitrile (70:30, v/v). The flow-rate was 1.0 ml/min.

Prior to analysis, 200 μl volumes of methanolic solutions of standards and samples were mixed with 400 μl of water. Aliquots (80 μl) were injected in duplicate onto the column. AMB was monitored at a wavelength of 405 nm and 0.02 absorbance units full scale (AUFS).

2.6. Quantitation

Standard calibration plots were constructed by least-squares linear regression of peak area on AMB concentrations. The values from the regression line were used for calculating the concentrations of AMB in the samples from their peak areas ratios. Final concentrations in BAS samples were calculated by multiplying the obtained concentration by a dilution

factor of two. Unknown concentrations of AMB in BAL samples were calculated by dividing the obtained concentration by the concentrated ratio; volume of BAL (μl)/400.

2.7. Analytical recovery and precision

The recovery study was carried out by comparing the peak area of the BAS- and BAL-spiked samples (0.1, 0.25, 0.5, 1, 2.5 and 5 μg/ml) to the respective non-extracted standards (AMB in methanolic solutions at the same concentrations).

Within-day RSDs was determined by performing ten solid extractions and assaying BAS and BAL samples of known concentrations (0.5 and 2.5 μg/ml), ten times in the same run. Day-to-day variation was calculated by assaying BAS and BAL samples of known concentrations (0.5 and 2.5 μg/ml), once a day for ten (BAS) and five days (BAL).

2.8. Stability

Sample stability at room temperature (20–25°C), 4°C and −20°C was determined by assaying BAS and BAL samples that had been spiked with 0.5 and 2.5 μg/ml of AMB. Aliquots for analysis of AMB were drawn at one, two, seven and fifteen days and at one, two and three months. A decrease of more than 10% from the initial concentration was considered to represent a significant loss of drug [13].

2.9. Patients

The procedure was evaluated in six patients who were treated with aerosolized AMB. The patients received a dose of 6 mg by inhalation. AMB was measured in BAS (n=12) and BAL (n=12) samples obtained 4 and 12 h after drug administration.

3. Results and discussion

Representative chromatograms of BAS and BAL specimens containing AMB are shown in Fig. 1. The retention time for the drug was 1.5 min. As we reported previously [11], the calibration curve was linear up to 5 μg/ml. The lowest detectable concentration at a signal-to-noise ratio of three was 0.05 μg/ml.

Results from the recoveries of the added substance are summarized in Table 1. The efficiency of the extraction was higher than 86% for all of the concentrations studied. The mean recoveries from BAS (n=30) and BAL (n=30) samples averaged 93.1 and 90.2%, respectively.

The within-day and day-to-day RSDs are presented in Table 2. The within-day RSD was less than 6% for BAS and BAL specimens. The day-to-day imprecision for BAS samples was less than 5%. The higher day-to-day variation was observed for BAL samples at a lower concentration (0.5 μg/ml). The lower reproducibility of the BAL samples compared to the BAS may be due to the fact that BAL samples suffered more manipulation. While the BAS is only submitted to methanol dilution, the BAL additionally undergoes solid–liquid extraction.

Table 3 shows the percentage changes in AMB concentration during the study compared with the initial concentration at time zero. As may be seen, the AMB concentration in the BAS fluid was maintained at between 90 and 110% of the initial concentration for two days at room temperature (20–25°C), fifteen days at 4°C and for three months at −20°C. The BAL samples were stable for one day at room temperature (20–25°C), seven days under refrigeration (4°C) and for one month at −20°C.

The median (range) AMB concentrations in BAS samples at 4 and 12 h after inhalation were 0.49 (0.45–5.66) and 0.53 (0.15–3.66) μg/ml, respectively. The median (range) BAL concentrations at 4 and 12 h were 18.64 (5.0–44.0) and 10 (5.44–31.66) μg/ml, respectively.

In conclusion, our results indicate that determination of AMB in respiratory secretions (BAS and BAL) by HPLC is a precise, sensitive and reproducible method that may be used for therapeutic monitoring and study of the pharmacokinetics of aerosolized AMB in clinical laboratories.

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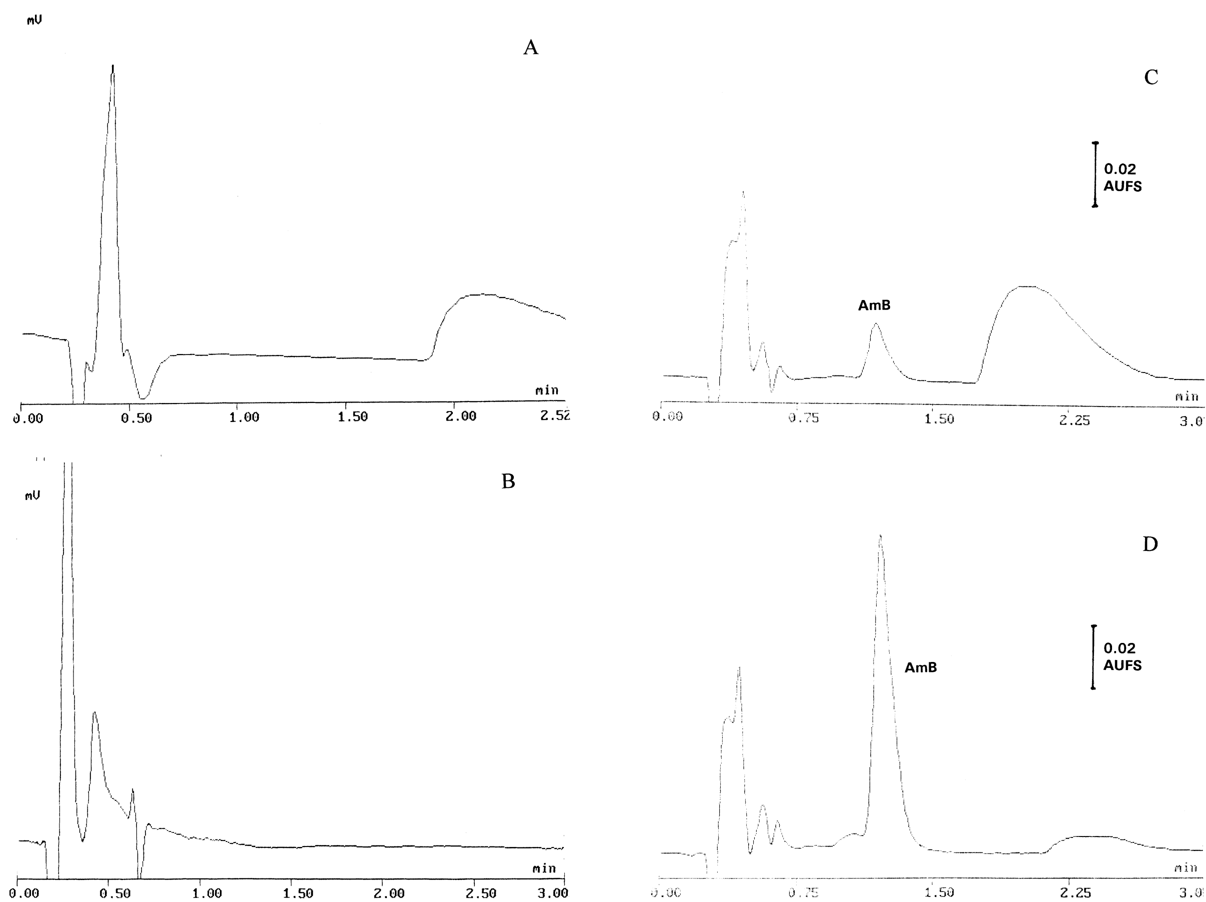

Fig. 1. Blank chromatograms of the (A) BAS and (B) BAL samples, and typical chromatograms of BAS and BAL samples containing (C) 0.57 and (D) 2.87 μg/ml amphotericin B (AMB).

Table 1
Recoveries of amphotericin B[a]

Amount added (μg/ml)	Recovery (%)	
	BAS (n=5)	BAL (n=5)
0.1	93.9±8.3	90.0±21.6
0.25	89.7±2.7	89.7±6.3
0.5	93.1±4.1	87.2±10.7
1	91.5±7.1	90.2±14.8
2.5	95.4±4.7	86.8±6.6
5	94.9±1.6	97.1±3.8
Mean	93.1±2.2	90.2±3.7

[a]Results are expressed as mean±SD.
n=30.

Table 2
Precision of the HPLC assay of amphotericin B

Sample	n	Concentration (μg/ml)			RSD (%)
		Added	Found		
			(Mean±SD)	Range	
Within-day					
BAS	10	0.5	0.52±0.029	0.47–0.55	5.58
		2.5	2.42±0.133	2.12–2.60	5.50
BAL	10	0.5	0.39±0.012	0.37–0.41	3.10
		2.5	2.32±0.110	2.10–2.48	4.74
Day-to-day					
BAS	10	0.5	0.51±0.021	0.48–0.54	4.12
		2.5	2.47±0.088	2.36–2.60	3.56
BAL	5	0.5	0.48±0.057	0.40–0.55	11.87
		2.5	2.42±0.175	2.20–2.56	7.23

Table 3
Stability of BAS and BAL samples containing amphotericin B

Temperature conditions (°C)	Initial time zero drug concentration (μg/ml)	% Initial drug concentration remaining						
		1 day	2 days	7 days	15 days	1 month	2 months	3 months
BAS								
20–25	0.53	98.8	95.1	85.9	87.1	85.3	82.1	68.5
4	0.53	97.7	97.6	99.2	99.8	84.5	84.7	75.6
−20	0.53	100.6	98.2	101.1	95.2	95.3	92.5	92.3
20–25	2.20	98.5	93.1	82.7	84.4	84.1	79.8	62.3
4	2.20	105.8	94.5	106.6	102.8	89.6	86.9	78.3
−20	2.20	107.0	103.8	104.7	104.6	104.8	103.1	95.1
BAL								
20–25	0.49	96.9	69.1	53.7	43.6	30.5	30.7	25.6
4	0.49	92.6	100.0	91.7	76.4	65.2	54.6	48.7
−20	0.49	92.6	91.3	95.3	91.8	94.7	82.8	76.3
20–25	2.49	94.4	87.9	77.4	69.4	63.9	43.9	39.2
4	2.49	98.4	93.3	94.5	61.4	30.8	36.1	32.3
−20	2.49	92.4	96.4	94.7	101.1	98.4	81.2	75.7

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