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Technical note

Determination of amphotericin B in human serum by liquid chromatography

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Abstract

A rapid reversed-phase high-performance liquid chromatographic method with a 30-mm long column is described for assaying amphotericin B in serum. After deproteinization of serum samples with methanol, the supernatant was injected onto a reversed-phase C_{18} column, using 2.5 m*M* Na_2EDTA–acetonitrile (70:30, v/v) as the mobile phase. Amphotericin B was eluted at 1.5 min. Calibration plot of the peak area against concentration was linear from 0.05 to 25 μg/ml (C.V. of 3%). Within-day and day-to-day imprecision (C.V.) ranged between 1.33% and 3.61%. The application was evaluated in 55 serum samples from patients treated with amphotericin B.

Keywords: Amphotericin B

1. Introduction

Amphotericin B (AMB) is considered the antibiotic of choice for a variety of systemic fungal infections. Long-term administration of AMB is associated with a high incidence of adverse effects, most commonly nephrotoxicity, and careful monitoring of patients under treatment is required [1].

Various methods have been described for AMB determination including high-performance liquid chromatography (HPLC) and microbiological assays [2–9]. The chromatographic methods reported in the literature use conventional columns (50–300 mm length) with retention times between 4 and 8 min.

This paper describes an isocratic reversed-phase (HPLC) method for the assay of AMB with a 30-mm long column. The procedure was evaluated in a clinical setting to determine its usefulness in monitoring serum levels in patients receiving AMB treatment.

2. Experimental

2.1. Standards and reagents

Sodium desoxycholate of AMB (Fungizone) was kindly supplied by Squibb & Sons (Princeton, NJ, USA). Ethylenediaminetetracetate disodium dihydrate ($Na_2EDTA \cdot 2H_2O$), methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). All chromatographic sol-

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vents were of HPLC grade, and all other chemicals were of analytical grade.

2.2. Instrumentation

The analysis was performed on a Kontron chromatograph 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 a 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. Chromatographic conditions

A column (30 mm × 4.6 mm I.D.) packed with Perkin-Elmer C_{18}, particle size 3 μm, was used. The mobile phase was 2.5 m*M* Na_2EDTA–acetonitrile (70:30, v/v). The flow-rate was 1.0 ml/min. A sample volume of 80 μl was injected into the column. The detector wavelength was set at 405 nm.

2.4. Sample preparation

The method described by Brassinne et al. [3] was used. The samples were deproteinized by adding 600 μl of methanol to 200 μl of serum, were mixed by vortex-mixing and centrifuged at 10 500 *g* for 5 min. The supernatant was injected by duplicate into the column. The standards were assayed in the same manner.

2.5. Calibration graphs

A stock solution of Fungizone (5 mg/ml) in water was prepared. Working standard solution containing 0.1–5 μg/ml of AMB was prepared in drug-free human serum by dilution of the stock solution. Deproteinization was done as described above. All the solutions were stored at −80°C until analysis.

2.6. Patients

Fifty-five samples were obtained from sixteen patients treated intravenously with a dose of 1 mg/kg/day of AMB, infused between 1 and 6 h. Serum samples were taken in steady-state conditions at 2 h, 6 h, 12 h and 24 h post-dose. Serum samples were stored at −80°C until assayed.

3. Results and discussion

Fig. 1 shows a typical chromatogram obtained with this procedure. The retention time for AMB was 1.5 min. The linearity was verified from 0.05 μg/ml (0.005 AUFS) to 25 μg/ml (0.2 AUFS).

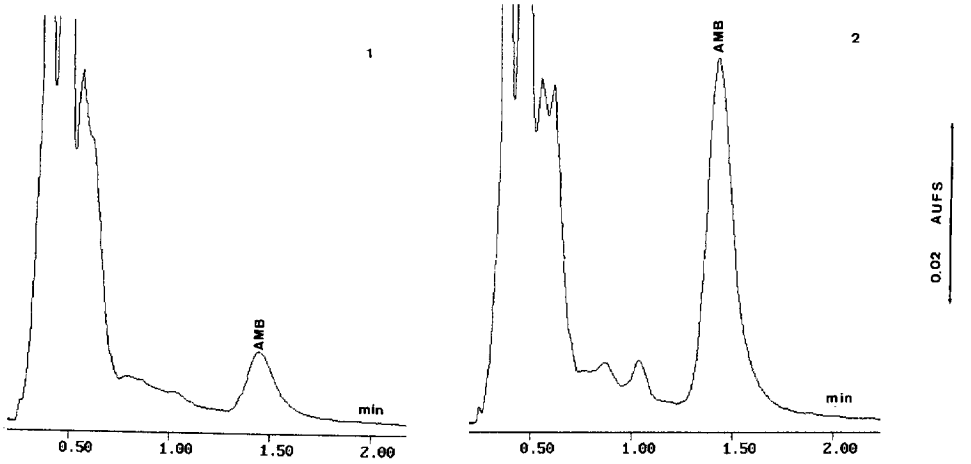

Fig. 1. HPLC profiles of amphotericin B (AMB) in serum from patients containing 0.45 μg/ml (1) and 2.43 μg/ml (2).

Table 1
Imprecision of the HPLC assay of amphotericin B ($n = 10$)

Concentration (μg/ml)				C.V. (%)
Added	Mean found	Range	S.D.	
Within-day				
0.5	0.43	0.42–0.46	0.01	2.40
2.5	2.23	2.17–2.28	0.03	1.33
Day-to-day				
0.5	0.46	0.44–0.49	0.02	3.61
2.5	2.56	2.45–2.70	0.09	3.60

The limit of detection at a signal-to-noise ratio of 3 was 0.05 μg/ml.

The regression equation between the peak-area (y) and the concentration (x) was $y = 43.103x + 0.031$ and the coefficient of variation (C.V.) was 3% ($n = 10$). Analytical recovery was determined by comparison of the peak area of AMB from deproteinized standard solutions to aqueous non-deproteinized standard solutions. The mean recovery for AMB was 90.2% ($n = 20$) over the range of 0.5 to 5.0 μg/ml. Within-day variation was determined by assaying each serum sample of a know concentration (0.5 and 2.5 μg/ml) ten times in a single day. Day-to-day variation was calculated by assaying serum samples once a day for ten days. The within-day and day-to-day coefficients of variation are presented in Table 1. Table 2 shows the serum AMB concentrations from 2 to 24 h post-dose, in the patients studied.

All the chromatographic methods previously described for the determination of AMB in biological fluids have utilized conventional columns [2–9]. The retention times described were between 4 and 8 min. The use of short columns (30 mm × 4.6 mm I.D.) presents a series of advantages over these techniques: fast equilibrium, less retention time, and lower consumption of the mobile phase; these factors are important in evaluating the practicability of the method. Although the sensitivity of our method is not as high as reported by others [7–9], it is sufficient for monitoring serum AMB concentrations along the dosage interval.

Table 2
Serum amphotericin B concentrations in sixteen patients treated intravenously with a dose of 1mg/kg/day of the drug

Hours post-dose	Concentration (μg/ml)		n
	Mean ± S.D.	Range	
2	1.13 ± 0.55	0.47–2.43	16
6	0.69 ± 0.36	0.13–1.61	15
12	0.53 ± 0.26	0.20–1.17	13
24	0.36 ± 0.17	0.14–0.83	11

4. Conclusion

Our results indicate that the short column reversed-phase liquid chromatographic analysis of amphotericin B provides a fast and inexpensive method for therapeutic monitoring in clinical laboratories.

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