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Note

High-performance liquid chromatographic determination of amphotericin B in human serum

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Amphotericin B (AmB) is a polyene antifungal compound that is still the drug of choice for the treatment of most severe systemic fungal infections. However, AmB is administered intravenously as a complex with sodium desoxycholate (Fungizone®) and induced many side-effects, especially renal dysfunction [1].

Recently we performed, in patients with resistant malignant tumours, a pilot trial of intravenous infusions of large volumes of sonicated liposomes containing a water-insoluble cytostatic agent [2]. We have used sonicated liposomes of the same composition — egg phosphatidylcholine, cholesterol and stearylamine (molar ratio 4:3:1; lipid concentration 20 mg/ml) — to entrap AmB. These liposomal preparations have been given intravenously, with an excellent tolerance, to cancer patients with suspected or proven fungal disease. None of the side-effects usually observed with Fungizone occurred [3].

To obtain data about the pharmacology of the liposomal preparation of AmB and to establish a possible relationship between serum levels and possible toxicity as well as clinical outcome, we needed a selective, sensitive and quantitative method for AmB determination in human serum. Microbiological assay is time-consuming and lacks sensitivity [4]. Several high-performance liquid chromatography (HPLC) assays have been published recently, but these techniques require many steps of preparation (deproteinization, centrifugation, filtration) [5,6] or have a very large coefficient of variation (C.V.) that precludes their use in pharmacokinetic studies of Fungizone, because this preparation gives rather low blood concentrations of AmB [7].

This report describes the application of reversed-phase HPLC to the determi-

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nation of the AmB concentration in human serum samples previously submitted to a quantitative extraction procedure.

EXPERIMENTAL

High-performance liquid chromatography

A Waters chromatograph was equipped with a Model 6000A solvent-delivery system, a Model 710B sample processor and a Model 440 absorbance detector operating at 405 nm; peak areas were integrated by a Waters Data Module M730. The stainless-steel column (30 cm×3.9 mm I.D.), prepacked with μBondapak C_{18} (average particle size 10 μm) was also supplied by Waters Assoc. (Milford, MA, U.S.A.). The column was preceded by an on-line stainless-steel precolumn (5 cm×3.9 mm I.D.) packed with Vydac-201 RP (particle size 30–40 μm; Macherey-Nagel., Duren, F.R.G.).

The mobile phase was methanol–acetonitrile–0.0025 *M* EDTA (500:350:200, v/v/v), delivered at 1.6 ml/min.

Reagents, standards and samples

HPLC-grade methanol and acetonitrile were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). EDTA was provided by Sigma (St. Louis, MO, U.S.A.). Desoxycholate AmB complex (Fungizone) was purchased from Squibb (Brussels, Belgium). AmB was a gift from Mr. P.V. Pigott from E.R. Squibb and Sons (Hounslow, U.K.).

A stock standard solution of Fungizone (5 mg/ml AmB) was prepared in doubly distilled water, and further dilutions were made with the mobile phase. Fungizone is the only available AmB preparation soluble in water.

Aliquots of the standard solutions were added to pooled human sera free of antimicrobial activity in order to obtain standard and control samples. Serum blanks were prepared in the same manner without AmB.

Blood samples obtained from patients treated with AmB-containing liposomes or with Fungizone were centrifuged at 1500 *g* for 10 min, and the serum supernatants were immediately extracted or frozen at −20°C until analysis.

Extraction procedure and conditions of analysis

The sample to be tested (0.5 ml) was mixed vigorously on a vortex mixer with 1.5 ml of methanol, left at 20°C for 30 min and centrifuged at 1500 *g* for 10 min at 20°C. The clear supernatant was decanted and injected into the liquid chromatograph. Table I lists the chromatographic conditions used in the analysis. All samples were extracted and chromatographed in duplicate, and the drug concentration was calculated from the peak area of AmB.

Typical chromatograms for a standard in mobile phase, a serum blank, a control sample and a serum sample from a patient are shown in Fig. 1.

TABLE I

CHROMATOGRAPHIC CONDITIONS

Parameter	Conditions
Column	μBondapak C_{18} (10 μm)
Mobile phase	Methanol–acetonitrile–0.0025 *M* EDTA (500:350:200, v/v/v)
Wavelength	405 nm
Flow-rate	1.6 ml/min
Temperature	Ambient
Chromatographic time	6 min
Chart speed	0.5 cm/min
Sample size	150 μl

RESULTS AND DISCUSSION

Analytical variables

The absorbance scan of AmB in the ultraviolet–visible spectrum shows four absorption maxima at 345, 363, 382 and 406 nm. The absorption at 406 nm is maximal, and at shorter wavelengths more methanol-extractable contaminant material is detectable in extracts of biological specimens.

Among the different extraction procedures tested, the best recovery is obtained

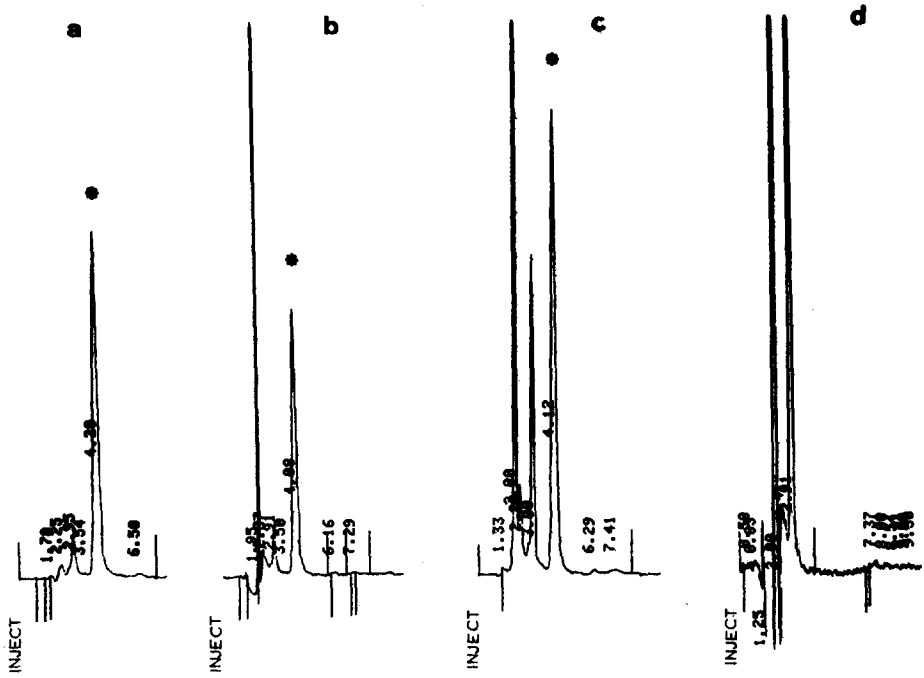

Fig. 1. HPLC profiles of AmB (*). (a) Standard in the mobile phase (0.05 a.u.f.s.); (b) human serum spiked with 1.0 μg/ml AmB (0.05 a.u.f.s.); (c) serum sample from a patient after infusion of liposomes containing AmB (0.2 a.u.f.s.); (d) blank human serum (0.005 a.u.f.s.).

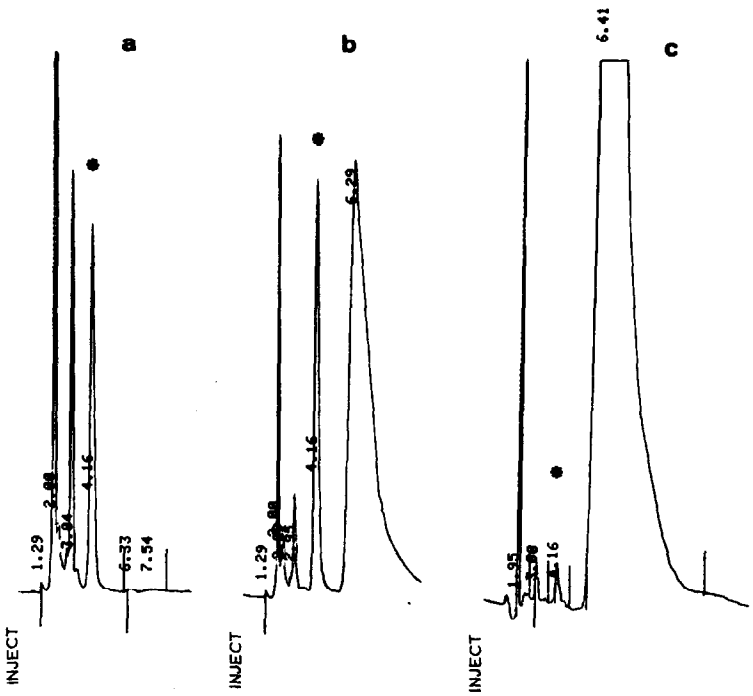

Fig. 2. HPLC profiles of AmB (*). (a) Serum sample from a patient with 8.1 mg/dl bilirubin (0.1 a.u.f.s.); (b) serum sample from a patient after bone marrow transplantation (0.2 a.u.f.s.); (c) hemolysed serum sample from a patient after Fungizone infusion (0.05 a.u.f.s.).

by methanolic extraction. The mixture must stand at least for 15 min at room temperature to obtain maximal precipitation of proteins and quantitative extraction of AmB.

The isocratic mobile phase must contain EDTA at a final concentration of 0.0005 *M*, since in its absence AmB peaks are neither quantitative nor reproducible.

Although Mayhew et al. [7] reported variations in the AmB recovery due to the use of a guard precolumn, we have not observed any effect on the analytical recovery yield of AmB by using a precolumn filled with Vydac-201 RP.

As shown in Fig. 1, in the chromatographic system used the elution profiles of standard (a) and extracted (b, c) serum samples are similar. In serum, the drug peak is well separated from other detectable compounds, and the extract of serum blank (d) shows no interfering peak at the AmB retention time.

Interfering peaks were reported in AmB assays of serum from patients with hyperbilirubinemia [5–7]. Fig. 2a shows the chromatogram of an extracted serum sample from a patient receiving AmB-containing liposomes and whose total bilirubin was high (5.8–13.6 mg/dl); no interfering peak is detected at the retention time of AmB.

As shown in Fig. 2 b, a well separated peak that elutes after AmB is detected in the extracted serum of a patient who has undergone a bone marrow transplantation, and who received AmB-containing liposomes. This peak seems to be due

TABLE II

RECOVERY STUDY

Amount of AmB added (μg/ml)	Amount of AmB measured* (μg/ml)	Recovery (%)
0.040	0.039	97.5
4.0	4.006	100.2
80.0	80.382	100.5

* Values represent the average of six separate determinations.

to red blood cell hemolysis, since it is also detected in a hemolysed serum sample from a patient receiving Fungizone infusions (Fig. 2c).

The patients treated with AmB-containing liposomes received associated treatments with one or several of the following drugs: vancomycin, erythromycin, ceftazidine, cotrimoxazole, furosemide, dopamine, methylprednisolone, amikacin, cimetidine, heparin, isoniazid, cyclosporin A, theophyllin, teicoplamin, timentin, ranitidine, rifampicine, metronidazole, digoxine. No interfering peaks were detected in the extracted serum samples obtained prior to infusion of the AmB-containing liposomes.

Linearity

There is a linear relation between the recorded peak height and the concentration of AmB in the standards prepared in the mobile phase ($r=0.9999$) and in the control serum samples ($r=0.9989$), extending to a concentration of 160 μg/ml AmB.

Recovery

Known amounts of AmB were added to drug-free serum to provide concentrations ranging from 0.04 to 80.0 μg/ml. After duplicate extraction and chromatography of six samples of each concentration, the peak areas obtained were compared with the peak areas obtained for working standard serum concentrations. Absolute recovery was calculated as: amount of drug measured/amount of drug added (μg/ml) $\times 100$. The results are summarized in Table II. A 97.5%–102.5% absolute recovery of AmB is obtained within the concentration range 0.04–80.0 μg/ml of serum.

Serum samples from patients were stored for twelve months at $-20°C$ and yielded concentration values identical with those obtained for the fresh serum sample.

Limit of quantitation

The limit of quantitation of the assay (i.e. peak height corresponding to twice the baseline noise) was found to be 0.01 μg/ml.

Precision

The between-day precision was calculated for seven concentrations of AmB in serum. Six samples of each concentration were extracted and chromatographed

TABLE III

BETWEEN-DAY PRECISION ($n=12$)

AmB concentration (μg/ml)	Coefficient of variation (%)
0.5	5.09
5.0	4.81
50.0	2.54

each day for twelve working days. The results (Table III) show that the method has a satisfactory precision with a C.V. equal to or less than 5.09% for AmB serum concentrations in the range 0.5–50.0 μg/ml.

Clinical applications

The method described in this paper is an accurate and reproducible procedure for the measurement of AmB concentrations in human serum. The sensitivity of the method makes it useful for pharmacokinetic and toxicological studies in patients treated with AmB administered as a liposomal preparation or as Fungizone. Fig. 3 shows the results of pharmacokinetic study. After one Fungizone infusion (1.2 mg/kg), the patient was treated with seven AmB-containing liposome infusions. After Fungizone infusion the peak serum level did not exceed 2.5 μg/ml, although after the first liposomal infusion of the same AmB dose, the peak

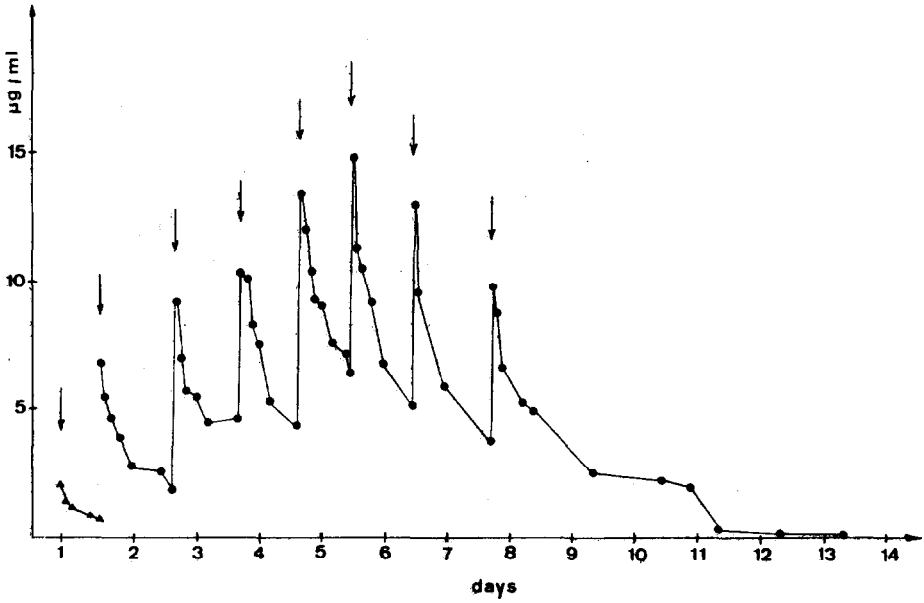

Fig. 3. Serum concentration profiles of AmB during and after intravenous infusion of Fungizone (▲), or AmB-containing liposomes (●). Arrow indicates start of the infusion.

value reached 7.0 μg/ml and values between 10.0 and 15.0 μg/ml were obtained for the following infusions. In addition, AmB was still present at a concentration above 2 μg/ml three days after the final AmB-containing liposome infusion.

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