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Note

Reproducible measurement of amphotericin B in serum by high-performance liquid chromatography in alkaline buffer

M. LECLERCQ, M. FOUILLIT and G. PANTEIX

C.E.R.N.I.P., Institut Pasteur de Lyon, 77 rue Pasteur, 69365 Lyon Cedex 07 (France)

and

R. GUINET*

Laboratoire de Mycologie et Microbiologie Industrielle, Institut Pasteur de Lyon, 77 rue Pasteur, 69365 Lyon Cedex 07 (France)

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Amphotericin B has been used as a chemotherapeutic agent for nearly 25 years and remains today the drug of choice for most systemic fungal infections [1—4]. The major limitation to the use of amphotericin B is the need for parenteral administration and the induction of serious side-effects. The renal side-effects are the most serious with renal vasoconstriction and tubular degeneration [1, 3].

The antimycotic properties of amphotericin are related to its ability to bind sterols, especially ergosterol, in the membranes of fungal cells [5, 6]. Membrane sterols are constituents of eukaryotic cells and this binding may contribute to the prolonged tissue retention of the drug. During intravenous therapy, 90% of amphotericin B disappears from the blood and only low levels are found in biological fluids other than serum. Moreover, the amphotericin B portion remaining in the blood is strongly bound to serum lipoproteins. But investigations providing a rational pharmacokinetic basis of amphotericin B treatment are scarce and several important phenomena are poorly understood [7, 8].

Monitoring the concentration in biological fluids and in tissues may, in theory, assist the management of an amphotericin B regimen. However, the assay of this product is difficult [9]. Microbiological assays are time-consuming, not sensitive and imprecise. Among several different physicochemical assays recently developed [10—14], high-performance liquid chromatography (HPLC) is rapid, specific and sensitive, and looks promising for the assay of amphotericin B in biological fluids as well as in tissue homogenates [3, 5, 15]. However, great variations can appear during the methanolic extraction, and the

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introduction of an internal standard recently proposed has not fully solved the problem [5, 15, 16]. We report here a sensitive, accurate and reproducible HPLC assay for amphotericin B using an original extraction procedure in an alkaline buffered medium.

MATERIALS AND METHODS

Reagents and standard

Methanol, dimethylsulphoxide (DMSO) and hydrochloric acid were purchased from E. Merck (Darmstadt, F.R.G.), ethylenediaminetetraacetic acid (EDTA) was obtained from Riedel-Dahaen (Hannover, F.R.G.). Amphotericin B was a gift of Squibb Labs. (Neuilly-sur-Seine, France).

A stock standard solution of amphotericin B (1 g/l) was prepared in DMSO while a working standard solution (10 mg/l) was prepared by dilution of the stock solution with the mobile phase. The stock solution was stored at −20°C; the working standard was stored at 4°C and its stability was verified by absorbance at 405 nm (E_m = 106,457).

Chromatography and extraction procedure

The analyses were carried out using a Waters high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a 6000A Waters pump and a U6K injector. The detector was a Waters Model 440 spectrophotometer with 405- and 340-nm filters, connected to an Omniscribe stripchart recorder (Houston Instruments, Austin, TX, U.S.A.). The column was a reversed-phase Waters Rad-Pak C_{18} (10 cm × 8 mm; 10 μm average particle size). The mobile phase was a mixture of methanol—5 m*M* EDTA (80:20), adjusted to pH 7.8 with dilute hydrochloric acid.

The mobile phase was added to serum in the proportion 3:1, vortexed for 2 min, kept at ambient temperature for 10 min and centrifuged at 2000 *g*. Then, 100 μl of supernatant were injected into the chromatograph.

Microbiological assays

The bioassays were performed according to a modification of the method of Shadomy et al. [17] using *Paecilomyces variotii* as test organism [18].

Human samples

Physicochemical (HPLC) and microbiological assays were simultaneously performed on 60 human sera from patients treated for deep mycosis with amphotericin B associated or not with 5-fluorocytosine.

RESULTS

Chromatography

Fig. 1 shows chromatograms of the working standard solution and of a human serum extracted as described in Materials and methods. Absorbance was simultaneously read at 405 and 340 nm and interfering substances were detected by measuring the ratio of absorbance at 405 nm to that at 340 nm.

Linearity and recovery

Different concentrations of amphotericin B in the range of concentrations of

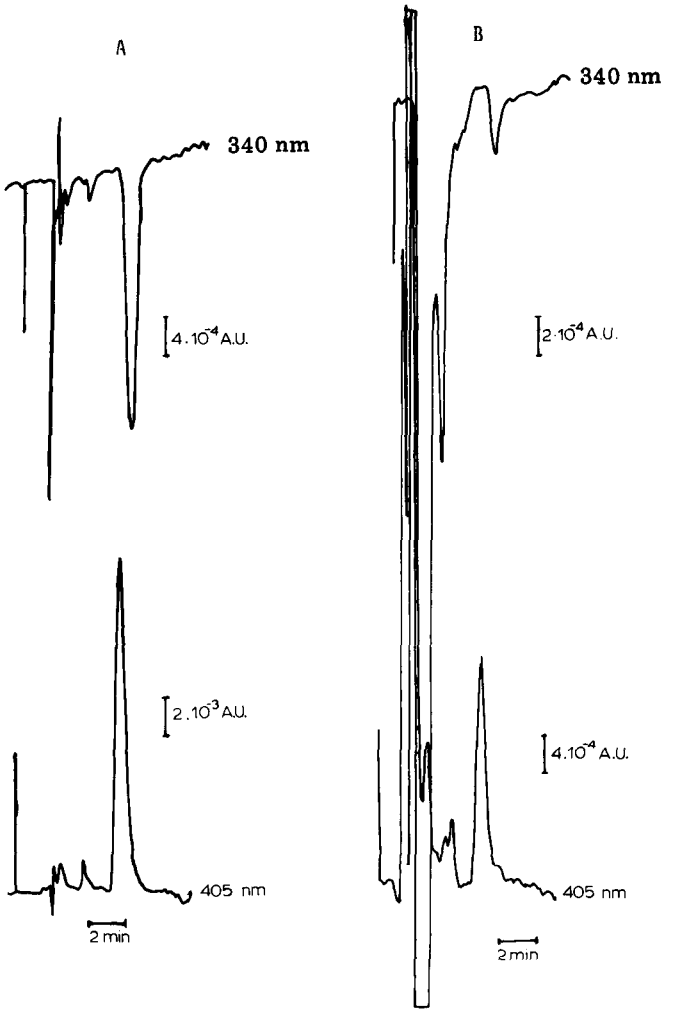

Fig. 1. Typical chromatograms of (A) extract of a standard solution containing 1.25 mg/l amphotericin B and (B) extract of serum sample containing 1.67 mg/l. A.U. = absorbance units.

TABLE I

LINEARITY AND RECOVERY STUDIES

Concentration added (mg/l)	Concentration recovered (mg/l)	Yield (%)
0.1	0.075	75
0.2	0.149	75
0.4	0.307	77
0.6	0.48	80
0.8	0.67	83
1.0	0.97	93
1.2	0.95	79
1.4	1.16	83

clinical interest were added to the same drug-free human serum. The concentration recovered and the yield of the assay are reported Table I. The mean yield of the assay was 81 ± 6%. The regression equation was $y = 0.838x \pm 0.001$ with a correlation coefficient of 0.993. The sensitivity of the assay, i.e. peak height corresponding to three times the baseline noise, was found to be 0.05 mg/l.

Precision

The internal coefficient of variation, by analysing the same serum ten times, was ± 3% (average concentration 1.55 mg/l).

Selectivity

Since amphotericin B is frequently used in association with 5-fluorocytosine

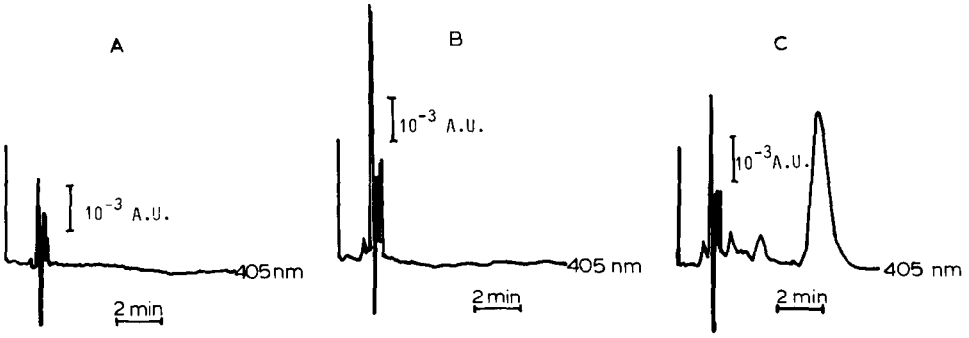

Fig. 2. Chromatograms of extracts of a normal serum sample with (A) no drug, (B) 10 mg/l 5-fluorocytosine, and (C) amphotericin B. A.U. = absorbance units.

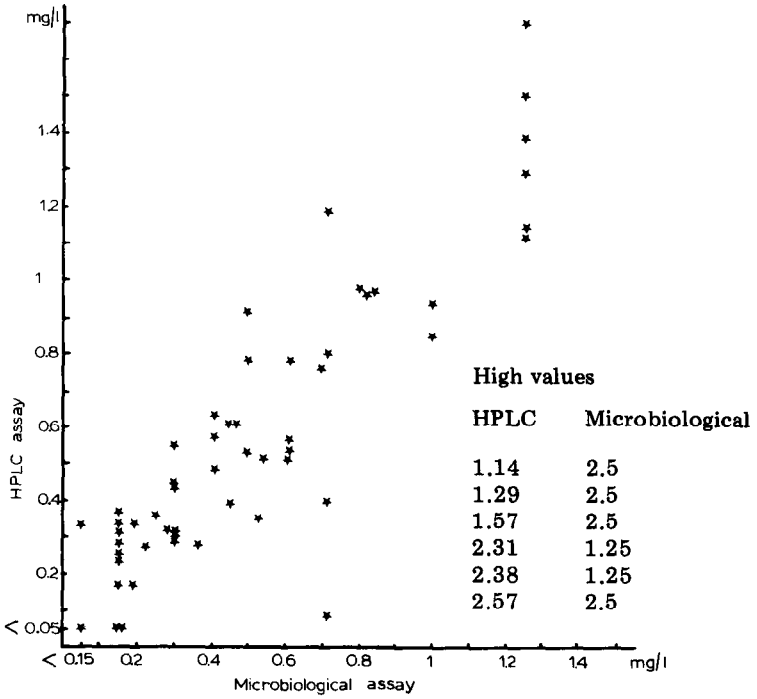

Fig. 3. Comparison of results of the microbiological assay and those of HPLC assay for determination of amphotericin B levels in 60 serum samples.

in the treatment of deep mycosis, we have verifyied that 5-fluorocytosine did not interfere with the amphotericin B assay. Fig. 2 shows a chromatogram of an extracted serum without any drug, and of the same serum with either 5-fluorocytosine or amphotericin B added. It is clear that 5-fluorocytosine does not interfere in the method.

Correlation of the physicochemical assay with the microbiological assays

The microbiological assays and the HPLC method were used simultaneously for 60 serum samples. The correlation coefficient calculated from the values represented in Fig. 3 was 0.7851 (regression equation $y = 0.2234 \pm 0.7008x$).

DISCUSSION

Nilsson-Ehle et al. [15] proposed a rapid and selective HPLC assay for amphotericin requiring many steps (deproteinization, centrifugation, filtration). However, Mayhew et al. [5], using identical experimental conditions, reported a fairly large coefficient of variation of 18% and an extraction yield varying between 53% and 71% within a concentration range of 0.08—10.0 mg/l.

Our previous results using this procedure gave an extraction yield from a standard solution of about 50% (Fig. 4). Furthermore, great variations were

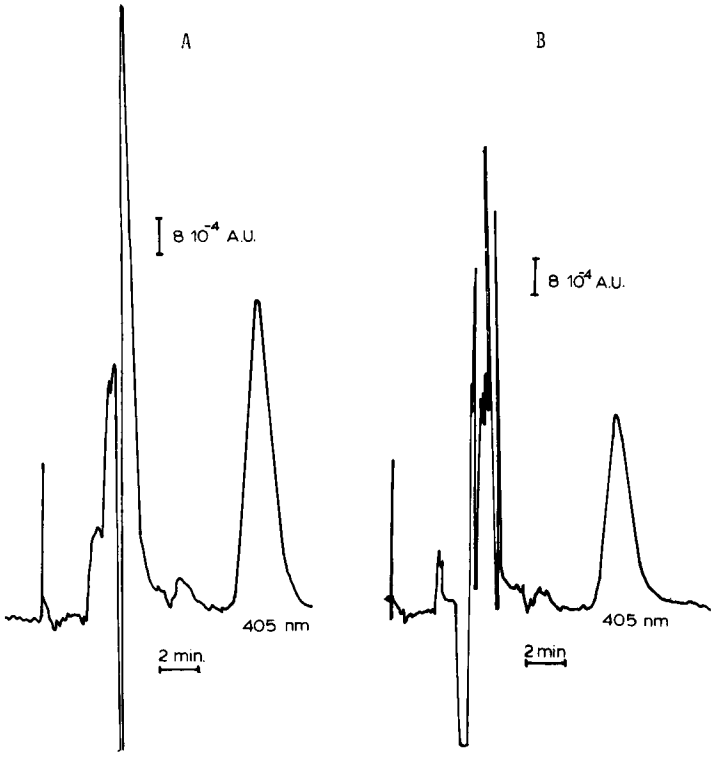

Fig. 4. Influence of pH of mobile phase and extraction buffer on the recovery. (A) Extract of 0.20 μg of standard solution with a mixture (80:20) of methanol—EDTA pH 7.8 as extraction buffer and mobile phase. (B) Extract of 0.20 μg of standard solution with a mixture (80:20) of methanol—water as extraction medium and mobile phase.

observed with patients, the HPLC results being in agreement with those of the bioassay only for some patients. It is well known (Merck Index) that the solubility of amphotericin B in water varies considerably with pH, being insoluble at pH 6—7 and soluble at 0.1 g/l at pH 2 and 12. We considered it important to use extraction and chromatographic conditions of pH compatible with amphotericin B solubility. We have retained pH 7.8 since a more alkaline pH could damage the column. Moreover, the procedure was simplified since we used the same alkaline buffer as mobile phase and for protein precipitation and amphotericin B extraction. Using the experimental conditions, the amphotericin B extraction step was satisfactory and reproducible (Fig. 4) and, since the recovery reached 80% (Table I), our assay was equivalent to previous results [15, 16]. Moreover, in contrast with Golas et al. [16], our HPLC method and the microbiological assays were simultaneously used for 60 patients' serum samples and a good correlation coefficient was obtained. Monitoring the concentration of amphotericin B in biological fluids is the usual way to adjust the drug regimen and it has been considered that we should produce, 1 h after infusion, a serum concentration of about twice the minimal inhibitory concentration of the fungus. However, the clinical usefulness of this approach has been discussed since increasing amphotericin posology was not always accompanied with increased serum concentrations. Most of the administered dose is bound to sterol-containing membranes in different tissues [4, 6, 8]. This rapid, sensitive and reproducible HPLC assay for amphotericin will allow us to determine tissue levels which may be more relevant for the clinical management of a patient [4, 8].

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