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Quantitation of amphotericin B in plasma by second-derivative spectrophotometry

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Abstract

A method is described for determining amphotericin B in plasma using second-derivative spectrophotometry after deproteinization. The assay was based on the absorbance at 407.5 nm. The second-derivative spectrum recorded between 350 and 450 nm allowed identification of the analyte and showed absence of drug interference. Only bilirubin interfered at high concentration (≥ 50 μmol l^{-1}). The linear concentration ranges were 0.05–5.0 mg l^{-1} ($r = 0.999$, slope = 2.731, intercept = 0.008). Between-day CV $\leq 9.7\%$, withinday CV $\leq 5.5\%$, analytical recovery close to 100% were suitable for clinical investigations. This method provides better specificity than direct absorbance, is simpler and faster than a high performance liquid chromatography assay and can be used routinely by any laboratory possessing a spectrophotometer with a derivative accessory. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Amphotericin B; Second-derivative spectrophotometry; Human plasma

1. Introduction

Amphotericin B, an antifungal antibiotic of the polyene family, is extracted from *Streptomyces nodosus* cultures. In addition to *Candida*, its activity spectrum includes fungi such as *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum* and the *Aspergillus* species. It is administered by slow venous infusion for the treatement of septicemic or visceral mycosis. No amphotericin B metabolite was identified [1,2]. The drug is excreted very slowly in the urine. Its administration includes a toxic risk for the kidney. Monitoring of blood concentrations is particularly required in patients with acute renal insufficiency.

Various assay methods have been reported, some using spectrophotometry with absorbance measurement [3] and others high-performance liquid chromatography (HPLC) [4–15]. The method described here, employing second-derivative spectrophotometry after simple extraction, is more specific than direct absorption measurement and faster than HPLC assay.

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2. Experimental

2.1. Sample collection

Blood was withdrawn in a heparin tube. As amphotericin B is a fragile molecule sensitive to light and heat [6], the tube was wrapped in aluminum foil and rapidly conveyed to the laboratory. After centrifugation, the assay was performed on plasma.

2.2. Apparatus and reagents

A Philips PU 8700 scanning spectrophotometer (Cambridge, Great Britain) with derivative accessory was used for the assay, the reagents being acetonitrile (Baker HPLC grade, Deventer, Holland) and dimethylsulfoxide (DMSO) (Merck, analytical grade, Darmstadt Germany). Amphotericin B was obtained from Sigma (St. Quentin Fallavier, France).

2.3. Preparation of standard solution

A stock standard solution (500 mg l^{-1}) was prepared in a mixture of DMSO and methanol (1:1, v/v). Immediately before use, this solution was diluted in distilled water to yield a working solution at 25 mg l^{-1}. Flasks containing these solutions were wrapped in aluminum foil.

2.4. Procedure

The calibration standards containing 0, 1.25, 2.50, 3.75 and 5.0 mg l^{-1} of amphotericin B were prepared by diluting the working solution with amphotericin B free human plasma. The standards and patient samples were both subjected to the deproteinization procedure: plasma samples (1 ml) was combined with 2 ml of acetonitrile and mixed by vortexing. After centrifugation at 5000 rpm at room temperature for 10 min, the supernatant was used for spectrophotometric measure. A reagent blank was prepared by addition of 2 ml of acetonitrile to 1 ml of distilled water. All tubes were protected from light.

2.5. Spectrophotometric assay

The assay was performed in 10-mm lightpath cuvettes with the reagent blank serving as reference. The spectrophotometer was programmed to generate the second-derivative scan between 350 and 450 nm. Quantification involved measurement of amplitude of the second-derivative at 407.5 nm.

3. Results and discussion

3.1. Spectrophotometric scan

Fig. 1 shows the evolution of the spectrum in function of plasma levels. The characteristic form of the spectrum provides the identification of amphotericin B. Plasma free of analyte gives a horizontal tracing, signifying the absence of interference. The amplitude was measured between the baseline and the minima at 407.5 nm. It was proportional to amphotericin B concentration. The amplitude could have been measured between a maxima and the minima at 407.5 nm. But this

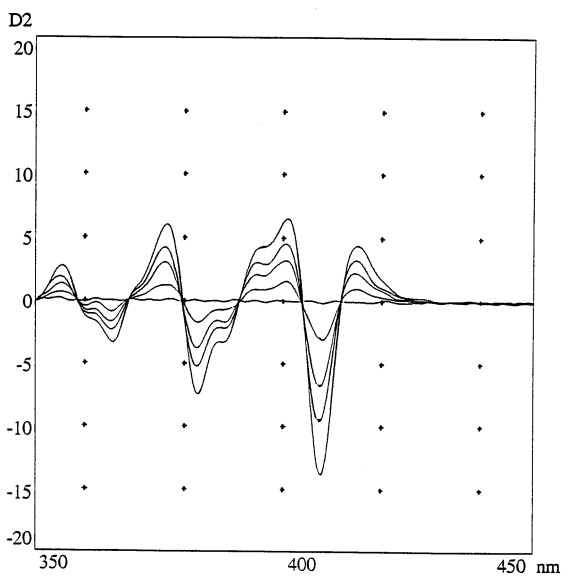

Fig. 1. Second-derivative spectrum of plasmas with amphotericin B added at different concentrations (0, 1.25, 2.50, 3.75, 5.0 mg l^{-1}).

signified two different measures at two wavelengths for each sample, with our spectrophotometer. As the method involving one amplitude measurement at 407.5 nm gave good results for linearity, precision and detection limit, it was chosen to be more practical. The amplitude measured was expressed in arbitrary units. Its absolute value was used to determine amphotericin B concentration.

3.2. Linearity

The linearity was assessed with concentrations from 0.05 to 5.0 mg l^{-1}. The between-day variability of the regression line was studied during 10 days. The composite regression equation was $y = 2.731x + 0.008$ (y = amplitude of second-derivative spectrum; x = concentration mg l^{-1}). The coefficients r were all > 0.999. The homogeneity of the results was demonstrated by the standard deviation of the slope 0.037. The standard deviation of the intercept was 0.077. When given in the usual therapeutic doses, amphotericin B plasma concentrations of 0.5 to 3.5 mg l^{-1} are measured [5].

3.3. Precision

Plasma samples spiked with amphotericin B at three different concentrations (0.50, 2.50 and 5.00 mg l^{-1}) were analyzed ten times in a single run (within-day precision) and ten times in separate runs over a period of two weeks (between-day precision). The method showed good precision with coefficients of variation (CV) of 1.2–5.5 and 1.2–9.7 respectively for within-day and between-day assay (Table 1).

3.4. Analytical recovery

It was determined by comparison of the concentrations in spiked plasma and distilled water. Results were $100.8 \pm 9.6\%$, $100.6 \pm 6.5\%$, $98.5 \pm 1.5\%$ and $98.7 \pm 4.4\%$ respectively for concentrations of 1.25, 2.50, 3.75 and 5.0 mg l^{-1}. Recoveries were satisfactory (all close to 100%).

Table 1
Amphotericin B assay precision

Plasma level	
Mean $\pm$ SD (mg l^{-1})	CV%
Within-day assay ($n = 10$)	
0.50 ± 0.03	5.5
2.54 ± 0.03	1.2
2.55 ± 0.07	1.3
Between-day assay ($n = 10$)	
0.52 ± 0.05	9.7
2.54 ± 0.03	1.2
5.16 ± 0.32	6.1

3.5. Interference

The absence of interfering compounds is verified by comparison of the second-derivative spectrum of plasma samples with that of calibration plasmas. In addition, a lot of plasmas or sera from patients treated with various drugs were tested: antiepileptic drugs (carbamazepine, valproid acid), antibiotics (vancomycin, amikacin, gentamicin, tobramycin, ceftazidime), antifungal agents (fluconazole, itraconazole), antidepressant drugs (maprotiline, clomipramine), theophylline. For flucytosin which is given to patients in association with amphotericin B, we spiked a plasma free of drugs. For all these substances, we did not notice any interference. As the measure was realized at 407.5 nm, in visible, we also tested drugs which could interfere in visible spectrophotometry. Such drugs are scarce. Nevertheless, plasmas of patients treated with idarubicin, epirubicin, methotrexate and rifampin were assayed. These drugs did not interfere. Concerning endogenous compounds, elevated concentrations of bilirubin disturbed the assay. A highly icteric plasma without amphotericin B produced a slightly modified scan. The modification occurred between 400 and 420 nm within the measurement zone (Fig. 2). Bilirubin interference occurred for the concentrations ≥ 50 μmol l^{-1}, corresponding to highly icteric plasmas. For icteric plasma we measure the absorbance at 430 nm, wavelength where we measure only bilirubin, and not amphotericin B (Fig. 3): Beyond a value of 0.350 absorbance units

(corresponding to 50 μmol l^{-1} of bilirubin), the second-derivative spectrophotometric assay at 407.5 nm could not be performed. To avoid bilirubin interference, the amplitude of the second-derivative spectrum could have been measured at 383 nm, other minima outside the modification zone. But this assay lacked sensitivity for low values. Various studies to eliminate bilirubin interference proved unsuccessful, whether by liquid–solid extraction on a Bondelut* (phenyl, CN, C8, C4, CBA) or Sep Pak* (C18) column or liquid–liquid extraction. A HPLC method [6] enabling bilirubin to be separated from amphotecin B was used for highly icteric plasmas. This alternative technique was rarely used since there were few icteric plasmas with a bilirubin concentration above 50 μmol l^{-1}.

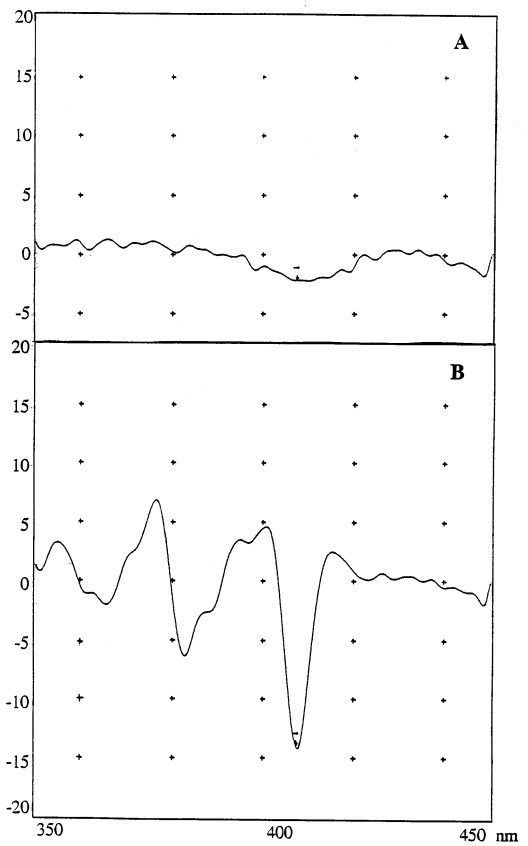

Fig. 2. Second-derivative spectrum of an icteric plasma (bilirubin = 210 μmol l^{-1}): free of amphotericin B (A); with amphotericin B added (5.0 mg l^{-1}) (B).

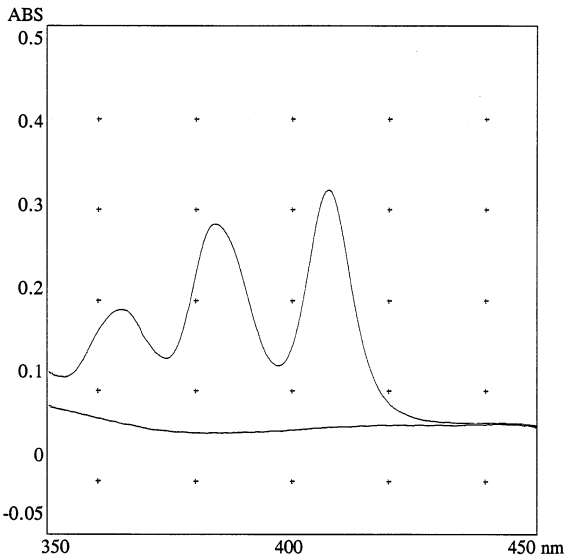

Fig. 3. Zero-order spectrum of a blank plasma and a plasma containing amphotericin B (5 mg l^{-1}).

3.6. Accuracy

Most HPLC assays use C18 reverse-phase chromatography with ultraviolet detection [4–10,12–15]. In a direct comparative study, 36 plasmas from patients treated with amphotericin B were assayed by both the HPLC method and second-derivative spectrophotometry. The chromatographic procedure was described by GOLAS et al. [6]. The concentrations determined by the two methods were compared with each other by linear regression analysis (Fig. 4) and showed good correlation. The correlation coefficient was 0.983, and the equation of the regression line was $y = 0.998x - 0.04$. HPLC methods are sensitive and specific but time-consuming: for a five samples serial range, the assay is achieved within 1 h for second-derivative spectrophotometry versus 2.5 h for HPLC.

3.7. Detection limit

It was defined as the lowest concentration detectable with reasonable certainty: the concentration corresponding to a 3SD signal from a plasma free of amphotericin B was 0.05 mg l^{-1} for the method described.

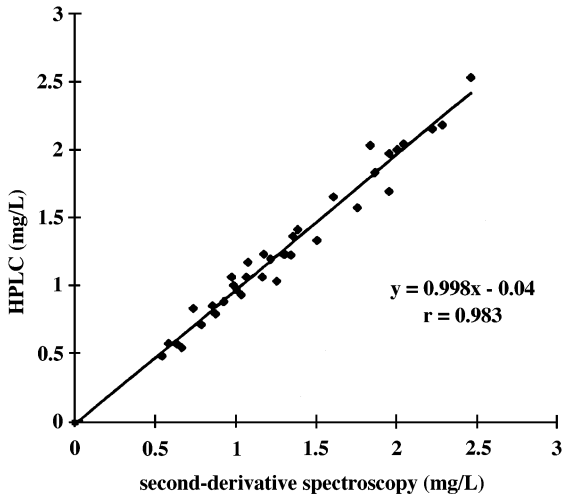

Fig. 4. Comparison of concentrations of amphotericin B in 36 patient plasmas assayed by HPLC and second-derivative spectroscopy.

3.8. Stability of the stock standard solution and samples

The amphotericin B stock standard solution at 500 mg l^{-1} in DMSO-methanol remained stable for at least 6 months at -20°C in darkness (flask wrapped in aluminum foil) [12]. The stability of plasma samples was tested at 4°C over 10 days and at -20°C for one month and showed no alteration during these periods. Golas et al. [6] have demonstrated that stability can be maintained over a 24-days period, whether at $+4$ or -20°C.

4. Conclusions

This study describes a simple, rapid and reliable method for an amphotericin B assay. Its good practicability makes it quite suitable for isolated or serial assays for treatment monitoring. The method can be easily applied in routine practice by any laboratory possessing a spectrophotometer with a derivative accessory.

The only drawback of the method concerns highly icteric samples. Interference from bilirubin at an elevated concentration cannot be eliminated and requires the use of a chromatographic assay in the rare cases encountered.

Second-derivative spectrophotometry is a considerable time-saver compared to HPLC, and the global cost of analysis is less than a chromatographic method.

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