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Improved high-performance liquid chromatographic determination of Amphotericin B in human serum and plasma

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Abstract: An improved reversed-phase high-performance liquid chromatographic procedure is described for the determination of Amphotericin B (AMB) in human serum and plasma. The procedure involves the addition of the internal standard, *p*-nitroaniline, to the sample (0.1 ml) followed by protein precipitation with acetonitrile. The supernatant is injected directly onto a C8 chromatographic column and eluted with an acetonitrile–aqueous 0.01 M sodium acetate buffer, pH 7.4, mobile phase. A spectrophotometric detector operated at 405 nm is used. Retention times for internal standard and AMB are 5.2 and 6.6 min, respectively. The assay standard curve is linear between 0.05–2.0 $\mu g\ cm^{-3}$. Within- and between-run relative standard deviations (RSD) for high and low concentrations of the drug are <5.30%. Analytical recovery of added AMB in serum is 98.4–101.4%. Data obtained by microbiological assay correlated well (r = 0.936) with LC results. Some commonly co-administered drugs and high concentrations of bilirubin are shown not to interfere.

Keywords: *Reversed-phase chromatography; Amphotericin B, assay in human serum and plasma; comparison with microbiological assay.*

Introduction

Amphotericin B (AMB), is a polyene antifungal agent which was isolated in 1956 from a soil actinomycete, *Streptomyces nodosus*, found in the Orinoco river area of Venezuela [1]. Nephrotoxicity and other side-effects [2], together with difficulty in establishing a clear relationship between blood levels of AMB and the balance between toxic effects and antifungal activity, indicate that clinical monitoring of this antibiotic should be undertaken. Consequently, clinical monitoring of AMB has been recommended [3].

Several methods for the determination of AMB have been developed. Microbiological techniques [4, 5] have been used widely for the determination of AMB in serum. Whereas these assays have proven reliable under routine, straightforward, clinical situations, they are of limited value when there is a need for rapid clinical feedback of the AMB levels and particularly where the patients involved have been subjected to multiple antifungal agents.

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Several high-performance liquid chromatographic (HPLC) methods [6–9] have been reported for the determination of AMB in serum and plasma. The results indicate that this technique would be useful for measurement of AMB in biological fluids because of its advantages over microbiological assays with regard to rapidity, accuracy and specificity. The most recently reported method [9] provides a good clinical assay for AMB, but it requires a solid-phase extraction procedure for the elimination of potential interference from conjugated bilirubin.

A new reversed-phase HPLC method for the rapid and precise determination of AMB in human serum and plasma without interfering bilirubin is now described. Results given by the LC method are compared with those given by microbiological assay.

Experimental

Liquid chromatography

The high-performance liquid chromatograph (Shimadzu Co. Ltd, Kyoto, Japan) used, consisted of a Model LC-6A solvent delivery system, a Model SIL-1A injector, and a Model SPD-6AV ultraviolet visible detector operating at 405 nm. The instrument was fitted with a 15 cm × 6.0 mm (i.d.) reversed-phase CLC-C8 column (particle size; 5-μm) supplied by Shimadzu which was operated at ambient temperature. The detector output was interfaced with a Model C-R4A (Shimadzu) computer which gave rapid automatic calculation based on the ratios of AMB/internal standard peak heights. Presumably, any similar means of calculating the data also would be satisfactory, including manual measurements.

Reagents and standards

The stock solution of AMB containing 1.0 g l^{-1} was prepared by dissolving 100 mg of AMB (a gift from Japan Squibb, Tokyo, Japan) in 100 ml of dimethyl sulphoxide, while a working standard solution (1.0 mg l^{-1}) was prepared by dilution of the stock solution with mobile phase. Serum controls, 0.1, 0.5 and 1.0 mg l^{-1}, were prepared by making appropriate dilutions of stock AMB in drug-free pooled serum. Aliquots (1.0 ml) of control serum, pipetted into disposable 13 × 35 mm polypropylene tubes and stored at −70°C, are stable for at least 6 months. A stock solution *p*-nitroaniline (obtained from Wako Pure Chemical Co., Osaka, Japan) containing 200 mg l^{-1}, was prepared using water as solvent. A working solution of internal standard 4 mg l^{-1} was prepared by diluting 2 ml of the stock *p*-nitroaniline solution to a total volume of 100 ml. Such a solution is stable for at least 6 months at −70°C. HPLC-grade acetonitrile and dimethyl sulphoxide were obtained from Katayama Chemical Co. (Osaka, Japan). All other chemicals were reagent grade. The mobile phase consisted of a mixture of 0.01 M sodium acetate buffer, pH 7.4 and acetonitrile (60:40, v/v) was delivered at a flow rate of 1.2 ml min^{-1}.

Procedure

A volume of 0.1 ml of 1.0 mg l^{-1} working standard, control serum, serum or plasma sample is pipetted into a 1.5 ml microcentrifuge tube. To each tube, is added 20 μl of the standard 4 mg l^{-1} solution of *p*-nitroaniline. Then acetonitrile (0.1 ml) is added to the solution which is vortexed for 15 s. After centrifugation at 12,000 *g* at room temperature for 2 min, a 50-μl aliquot of the supernatant is injected directly into the chromatograph.

Determination of the quantity of AMB in samples or controls is realised by dividing

the peak–height ratio of AMB to internal standard by the mean of the same ratio for duplicate determinations of standards.

Microbiological assays

The bioassays were performed according to a modification of the method of Shadomy *et al.* [4] using *Paecilomyces variotii* as the test organism.

Results

Retention times for internal standard and AMB under the present experimental conditions are 5.2 and 6.6 min, respectively. Representative chromatograms of a drug-free serum sample, a drug-free sample with added AMB (0.5 mg l^{-1}) and the internal standard, and a serum sample obtained 12 h after i.v. administration of AMB, 1 mg h^{-1}, to the patient with hyperbilirubinemia, respectively, are shown in Fig. 1. Total chromatography time for AMB is <8 min per run.

Peak–height ratios of AMB to internal standard are linearly related to AMB concentration in human serum over the range 0.05–2.0 mg l^{-1} (n = 8, correlation coefficient 0.999). A detection limit of 0.01 mg l^{-1} (signal-to-noise ratio 2:1) is achievable by the method.

Analytical recoveries of added AMB from serum by the extraction procedure (mean ± SD, n = 6) were found to be 98.4 ± 2%, 101.4 ± 1% and 99.4 ± 2% at concentrations of 0.1, 0.5 and 1.0 mg l^{-1}, respectively.

In order to define the within- and the between-run precision of the LC method for serum specimens, each of the three reference samples was analysed 10 times in a single run and six times in separate runs, the results being summarized in Table 1. The relative

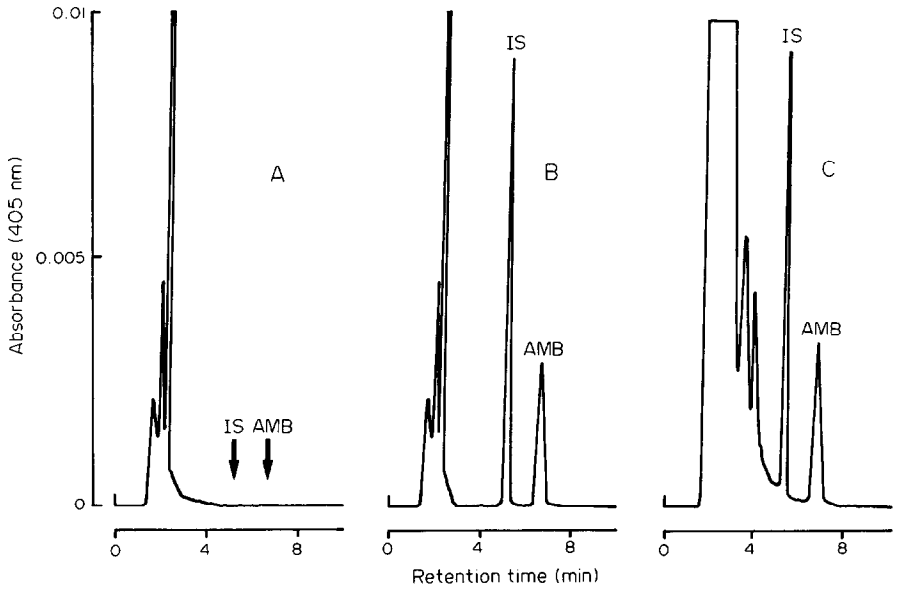

Figure 1
Representative chromatograms of extracted drug-free serum sample (A), a sample with internal standard (IS) and AMB (0.5 mg l^{-1}) (B), and a patient's serum sample containing AMB (AMB concentration was 0.56 mg l^{-1}, total bilirubin was 135 mg l^{-1}) and added internal standard (C).

Table 1
Precision data for the determination of AMB by HPLC

Expected concentration (mg l^{-1})	Observed concentration (mg l^{-1})					
	Intra-assay (n = 10)			Inter-assay (n = 5)		
	Mean	SD	RSD (%)	Mean	SD	RSD (%)
0.1	0.107	0.004	3.738	0.095	0.005	5.263
0.5	0.492	0.015	3.049	0.483	0.016	3.313
1.0	1.053	0.021	1.994	1.047	0.032	3.056

Table 2
Drugs found not to interfere with detection of AMB by HPLC

Antibiotics	Antineoplastic	Other drugs
Amikacin	Adriamycin	Acetaminophen
Benzylpenicillin	Allopurinol	Aspirin
Carbenicillin	Cisplatin	Cyclosporin A
5-Fluorocytosine	Cyclophosphamide	Phenobarbital
Gentamicin	5-Fluorouracil	Phenytoin
Ketoconazole	Methotrexate	Theophylline
Miconazole	Thioguanine	

standard deviations (RSD) ranged from 1.994 to 3.738% for within-run precision, and from 3.056 to 5.263% for between-run precision.

Twenty common drugs were tested for potential analytical interference (Table 2). These compounds were selected because they were either commonly used in clinical practice or were co-administered to the patients during clinical studies. All of the substances tested are eluted before the internal standard and AMB and did not interfere with the quantification of AMB. Additionally, no interference was obtained from icteric, haemolytic or lipemic samples.

Fifty-five clinical specimens obtained from 30 patients treated with AMB were assayed by HPLC and microbiological assay. There was a high degree of correlation between the drug levels detected by the two methods, as illustrated by Fig. 2. The correlation coefficient was 0.936, and the equation of the regression line was $y = 0.86x - 0.002$.

Discussion

Granich *et al.* [9], in their detailed study of isolation methods for AMB prior to liquid chromatographic analysis, indicated that removal of serum proteins with methanol was not suitable for samples containing high bilirubin levels (>30 mg l^{-1}) since extraneous peaks interfered with either the drug or internal standard peaks. They suggested that the conjugates of bilirubin in human serum account for the majority of this interference. They used a solid-phase extraction procedure which removed this interference. The simple sample preparation described in the present communication, effectively removes these bilirubin conjugates from the sample, while providing good recovery and precision for both internal standard and AMB.

Comparative experiments indicated a good correlation (r = 0.936) between HPLC and microbiological assays for AMB in serum; this shows that there is no major active metabolite interference in the serum. Although microbiological assays are more widely

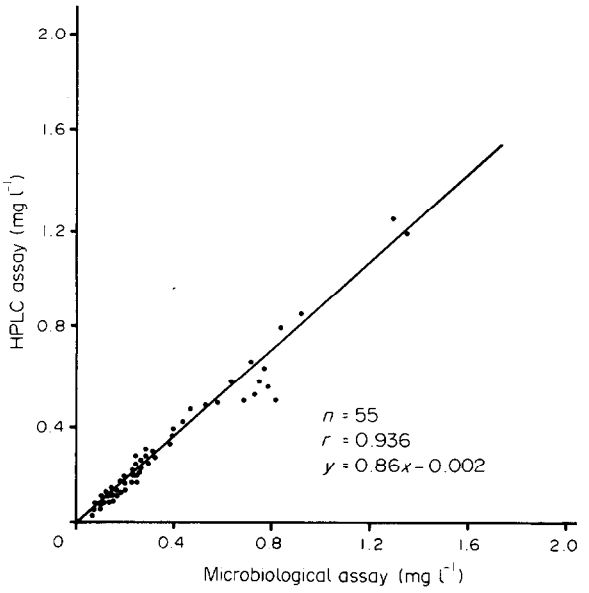

Figure 2
AMB as measured in the same patient's serum by the present method, compared with the results of microbiolgoical assay. Samples were routine clinical specimens, not AMB-supplemented serum.

used, they have their share of disadvantages. The HPLC procedure, including sample pretreated, can be completed in 10–15 min; on the other hand, the microbiological assays are far more time-consuming. When an antibiotic has active metabolite(s) or active decomposition products, or when two or more antibiotics are administered, the highly specific HPLC methods are to be prefered. The assay was designed to measure theraperutic concentrations ranging from 0.05 to 2.0 mg l^{-1}. When injection volumes of 50 μl are used, the lower limit of detection is 0.01 mg l^{-1} for the AMB.

The HPLC assay of AMB in serum and plasma is an accurate, rapid, sensitive and selective technique that is appropriate for use in clinical laboratory.

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