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High-Pressure Liquid Chromatographic Assay of Bay n 7133 in Human Serum

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A high-pressure liquid chromatographic method that includes a Sep-Pak (Waters Associates, Inc., Milford, Mass.) preparation of human serum was employed for the quantitative assay of Bay n 7133. Drug levels of 0.1 to 20 μg/ml could be detected. No interference from amphotericin B was found in the chromatographic analysis of Bay n 7133.

Bay n 7133 (Miles Pharmaceuticals, West Haven, Conn.) is a new investigational antifungal agent. In vivo and in vitro studies require an accurate and reliable assay method. Biological methods have been used (4) but are generally less precise than chemical methods such as gas chromatography or liquid chromatography (LC). The possibility of combination antifungal therapy favors the use of such chemical methods as opposed to a biological system in which other drugs may interfere in the quantitation of a single agent (5). The application of LC methodology was desired to expand the assay capabilities. An existing LC method for the antifungal agent ketoconazole (1) was tested for the assay of Bay n 7133 in human serum. The purpose of this report is to (i) describe the use of LC for the assay of Bay n 7133, including the sensitivity, precision, linearity, and drug recovery of this assay; (ii) describe the use of this assay in sample analysis from a patient receiving this agent in combination with amphotericin B; and (iii) compare the LC assay with biological assay.

MATERIALS AND METHODS

Extraction technique. Bay n 7133 was supplied by Miles Pharmaceuticals. Sep-Pak (Waters Associates, Inc., Milford, Mass.) C18 cartridges are small disposable units in which the most-polar sample components elute first. Preparing serum samples by this method removes proteins and polar interferences which would hinder liquid chromatographic determinations. Cartridges were prewet with 5.0 ml of methanol and washed with 5.0 ml of distilled water before use. Cartridges were alkalized with 0.2 ml of 0.1 M sodium carbonate and then a 1.0-ml serum sample was added. Serum proteins were washed out of the cartridge with 5.0 ml of distilled water. Next, 0.2 ml of methanol was pushed in via syringe to displace the water. Bay n 7133 was removed from the cartridge with 2.0 ml of methanol. This mixture was then injected directly into the chromatographic equipment. Standards or samples containing 0.1 to 0.5 μg/ml were partially evaporated under an air stream for ca. 30 min and then brought to a 1-ml volume with methanol to increase assay sensitivity before injection for quantitation.

LC assay. The chromatographic system used was a model LC 5020 liquid chromatograph (Varian Associates, Walnut Creek, Calif.) with a Varichrom variable-wavelength detector and a CDS 111L peak integrator with a strip chart recorder. An analytical reverse-phase μBondapak C_{18} column (30 cm long, 3.9 mm in internal diameter, and 10 μ in particle size; Waters Associates, Inc.) was used. The mobile phase was 30% 0.02 M KH_2PO_4 buffer, pH 7.5, and 70% methanol (vol/vol) at a flow rate of 2.0 ml/min. The detector monitored column eluate at 231 nm by using 0.1 absorbance units, full scale (AUFS); for samples of <0.5 μg/ml, 0.05 AUFS was used. A 100-μl injection volume for samples was used by means of a fixed-loop injector.

Microbiological assay. Assay plates were made with Biggy agar (Difco Laboratories, Detroit, Mich.) prepared according to package direction. This agar requires gentle heating and is not autoclaved. Heated agar was dispensed in 40-ml volumes into petri dishes (150 by 15 mm) and allowed to harden on a level surface. The moisture contents of plates were allowed to stabilize for 1 week at 4°C. *Candida pseudotropicalis* strain Carshalton (Miles Pharmaceuticals, West Haven, Conn.) was used as the indicator organism. A 48-h culture was used to make a suspension of organisms in sterile saline. This suspension was adjusted to a concentration of ca. 2.5×10^5 CFU/ml. Biggy agar plates were then flooded with organism suspensions, and subsequently emptied, and left at room temperature on a level surface to dry.

Six wells, 8 mm in diameter, were punched in each plate, and the agar plugs were removed. One hundred microliters of each specimen or standard was inoculated into each well. Each specimen was run on four plates, along with all three standards (0.3, 1.0, and 3.0 μg/ml). Sample placement on each plate was randomized owing to the variability of inoculum density over the surface of the agar plates. After overnight incubation at 35°C, zone diameters were read to the nearest 0.5 mm and averaged. A standard line was constructed on a semilog graph, with the abscissa representing average zone diameter and the ordinate representing standard concentration. Specimens with zone diameters larger than the 3.0-μg/ml standard was diluted with serum and repeat tested.

Samples. Serum samples for methods development and standards were prepared by using pooled human serum to which Bay n 7133 had been added. Bay n 7133 was dissolved in 1 ml of dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.), deionized water was added to a stock concentration of 1.0 mg/ml, and the mixture was further diluted in serum. Samples were spiked to contain concentrations of 0.1 to 20 μg/ml in human serum for LC assay analysis. Concentrations between 10 and 1.0 μg/ml were made to compare LC and bioassay systems. Venous blood was also obtained from a patient receiving Bay n 7133 therapy and was allowed to

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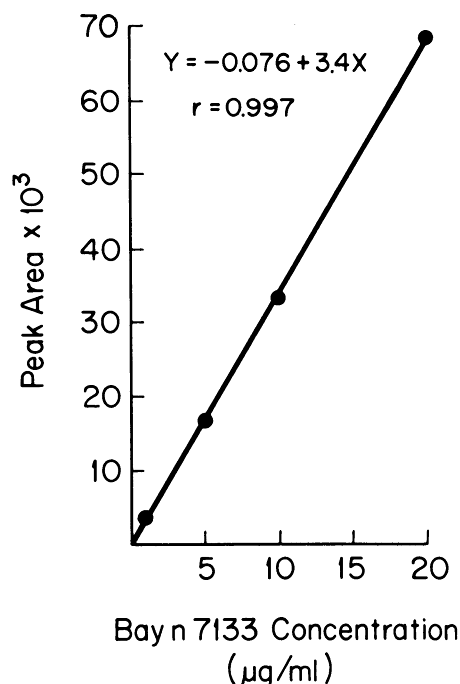

FIG. 1. Detector response of Bay n 7133 at 231 nm.

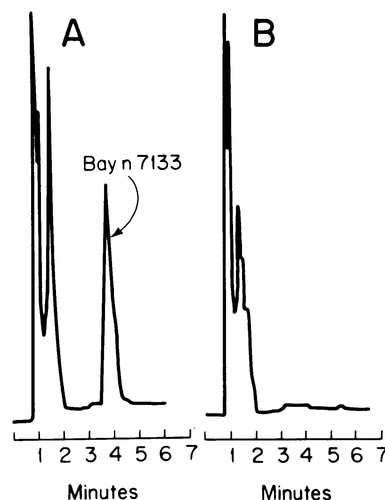

FIG. 2. Human serum chromatograms. (A) Extracted human serum with 10 µg of Bay n 7133 per ml; (B) blank extracted human serum.

clot, and serum was removed after a 10-min centrifugation at 550 × *g*. Patient samples were frozen at −80°C until assayed. Serum samples used in drug recovery calculation were spiked to a concentration of 5 µg/ml and then processed through Sep-Pak C18 cartridges. Water was spiked to a concentration of 5 µg/ml and diluted 1:2 with methanol to mimic the dilution of sample (1 ml of serum placed through a cartridge and eluted by 2 ml of methanol) in the preparation procedure. Peak areas from serum samples were divided by the peak area of water dilution to determine percent drug recovery.

RESULTS

The quantitative drug recovery with the Sep-Pak cartridge for sample preparation was tested on three separations which demonstrated a mean (range) recovery rate of 69 (65 to 73). The UV spectrum of Bay n 7133 had two absorbance peaks at 231 and 276 nm. Detector response at 231 nm was linear through the ranges tested, 0.5 to 20 µg/ml at 0.1 AUFS and 0.1 to 1.0 µg/ml at 0.05 AUFS (Fig. 1). Comparison of drug concentrations from extracted samples versus peak areas gave a correlation coefficient of 0.997 and a linear regression line $y = -0.076 + 3.4x$. Quantitation was sensitive to at least 0.5 µg/ml when 100 µl-injections and 0.1 AUFs were used and sensitive to at least 0.1 µg/ml when 0.05 AUF and sample concentration were used. Results from the analysis of 10 serum samples (5 µg/ml) had a coefficient of variation of 9.6%, which determines the combined precision of the LC assay system and Sep-Pak sample preparation. Bay n 7133 had a rentention time of 3.7 min. Using Sep-Pak preparation, we found no peaks in pooled human serum to interfere with the drug assay (Fig. 2).

Comparison of LC and microbiological assays at four drug concentrations gave a correlation coefficient of 0.986 and a linear regression line $y = -0.09 + 0.96\times$. The results of the LC assay were comparable to those of the microbiological system, as demonstrated by the slope of 0.96.

Six samples from the patient on concomitant amphotericin B therapy were tested for levels of Bay n 7133. Amphotericin B measured by high-pressure LC (6) in these samples ranged from 0.6 to 0.7 µg/ml. This patient was also receiving acetaminophen, diphenhydramine hydrochloride, dipyridamole, methadone hydrochloride, methylprednisolone, nystatin, phenytoin sodium, and trimethoprim-sulfamethoxazole. Four of the six patient samples had no detectable levels of Bay n 7133; these samples showed no other peaks near the retention time of Bay n 7133. The results of drug analysis are shown in Table 1. Three samples from a patient receiving Bay n 7133 and not amphoterecin B were assayed by both bioassay and LC (Table 2).

DISCUSSION

The LC assay described above affords a rapid sample preparation, along with a specific and quantitative method for Bay n 7133 measurement. The LC assay compared favorably with the bioassay, as was demonstrated by the results of the analysis of three patient samples and the good statistical correlation between the two methods. Concomitant amphotericin B administration did not create any interference, likely owing to the different wavelength maximums of the two compounds. 5-Fluorocytosine is assayed on a cation-exchange column (2) and, in our experience, is not retained on a C18 LC column; therefore, 5-fluorocytosine should not interfere in this assay. Several other drugs which may be encountered in patient samples also showed no

TABLE 1. Concentration of Bay n 7133 in serum of a patient also receiving amphoterecin B

Bay n 7133 dosage (mg)	Concn (µg/ml) in serum at time (h) after dose				
	0	1.5	2.5	3.25	4.5
400	0	—[a]	—	0	0
800	0	7.4	6.5	—	—

[a] —, No sample.

TABLE 2. Concentration of Bay n 7133 in serum of a patient after an 800-mg oral dose as determined by two methods

Time (h) after administration	Concn (μg/ml) in serum as detected by:	
	Biological assay	LC assay
24 (Trough)	0	0
1.25	0	0
2.5	1.8	1.6

interference. The retention of ketoconazole when 70% methanol and 30% (vol/vol) 0.02 M KH_2PO_4 buffer (vol/vol) was used was 5.5 min, making quantitation of both drugs in one sample possible. The precision of Sep-Pak preparation reported by Hildebrandt and Gundert-Remy (3) was between 1.5 to 6.7% depending on concentration, with the lower concentrations tending to have higher coefficients of variation. Precision of the Bay n 7133 Sep-Pak preparation was tested at a concentration at the probable midrange of sample levels and was 9.7%. Drug recovery was slightly less than that reported for ketoconazole (77%) by Andrews et al. (1). Quantitation of Bay n 7133 below 0.5 μg/ml can be achieved by increasing detector sensitivity, concentration of the sample, or both if required. The LC assay developed in these studies is a simple and accurate method for Bay n 7133 measurement in serum.

ACKNOWLEDGMENTS

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