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Simultaneous Determination of Arabinitol and Mannose by Gas-Liquid Chromatography in Experimental Candidiasis

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A method is described for the simultaneous quantitation of D-arabinitol and D-mannose in serum by gas-liquid chromatography as an aid for the diagnosis of disseminated candidiasis. Both variables were observed as per-*O*-acetylated aldononitrile derivatives in each chromatographic run of sera from immunosuppressed rabbits experimentally infected with *Candida albicans* 3181A.

Disseminated candidiasis is an important cause of morbidity and mortality in leukemic patients. Autopsy data have shown that the incidence can be as high as 34%. (8). Increasingly aggressive chemotherapy results in prolonged leukopenia and increased susceptibility to infection. Because of the immunosuppression, tests for antibody to *Candida* species are frequently negative among leukemic patients (2). On the other hand, sera from patients colonized with species of *Candida* frequently give positive results in conventional serological tests. Because of these shortcomings, tests that directly detect the antigens or metabolites of the *Candida* species have received considerable attention. Among these, three methods have been independently reported: enzyme immunoassay for mannanemia (5, 11), gas-liquid chromatography (GLC) for detection of D-arabinitol (1, 3, 10, 14, 15), and GLC detection of D-mannose (4, 6, 7). Although all three techniques seem to be promising, methods for the simultaneous detection of arabinitol and mannose have not been described. Because GLC is a time-consuming and specialized technique for the clinical laboratory, we wanted to be able to observe both D-arabinitol and D-mannose in each chromatographic run. We considered it especially pertinent to gather enough data about *Candida* species metabolites to build confidence that one or both of these variables are valid indicators of infection. The goals of the present study were therefore (i) to develop a disseminated candidiasis model in immunosuppressed rabbits and (ii) to devise a GLC method for the simultaneous quantitation of serum arabinitol and mannose.

(Some of these results were presented earlier [L. de Repentigny and E. Reiss, Program Abstr. Intersci. Conf. Antimicrob. Agents and Chemother. 22nd, Miami Beach, Fla., abstr. no. 487, 1982].)

New Zealand White rabbits (3 to 4 kg) were obtained from the Veterinary Services Branch, Centers for Disease Control. Four animals were immunosuppressed with daily injections of cortisone acetate (10 mg/kg per day, subcutaneously), beginning 2 days before and ending 7 days after infection. Two of these animals also received a single dose of cyclophosphamide (40 mg/kg, subcutaneously) 2 days before infection. After a base-line bleeding, animals were infected intravenously with 10^7 blastoconidia of *Candida albicans* 3181A, a serotype A human isolate obtained in 1971 and stored lyophilized at −40°C. This inoculum was prepared by suspending a 24-h slant culture on Sabouraud glucose medium with 3 ml of 0.9% NaCl, counting the suspension in a hemacytometer, and diluting it to yield 10^7 cells per ml. The number of viable cells injected was confirmed by serially diluting the inoculum cell suspension and plating it on Sabouraud glucose medium. Blood samples were then drawn every 2 days until the animals died. Moribund animals were sacrificed by exsanguination. Disseminated candidiasis was demonstrated by quantitative culture and histopathology of the kidneys and the liver. Quantitative culture was done by homogenizing the tissue in 5 ml of phosphate-buffered saline (pH 7.2) and serially diluting the homogenate and plating it on Sabouraud glucose medium. A sample of each organ was also placed in 10% buffered Formalin (pH 7.0) and stained by the hematoxylin and eosin, Gomori methenamine silver, and periodic acid-Schiff procedures.

Simultaneous quantitation of serum arabinitol and mannose was done as follows. First, 1.5 ml of acetone and the internal standard methyl α-D-mannopyranoside were added to 0.4 ml of unhydrolyzed serum. After centrifugation at 1,500 × *g* for 5 min, the supernatant was dried under nitrogen. A per-*O*-acetylated aldononitrile deriv-

ative (12) was prepared by adding 2 mg of hydroxylamine hydrochloride (Alfa Products, Danvers, Mass.) in 0.12 ml of dry pyridine (SpectrAR grade; Mallinckrodt, Inc., Paris, Ky.) to the dried sample. The mixture was kept at 60 to 65°C for 20 min in a module heater (Scientific Products Div., McGaw Park, Ill.) mounted on a variable-speed rotator and then cooled to room temperature. Acetic anhydride (0.08 ml) (AnalaR grade; BDH Chemicals Ltd., Poole, England) was then added, and the solution was again heated on a variable-speed rotator at 60 to 65°C for 20 min. The derivative was extracted with 1 ml of chloroform (99 mol% pure; Fisher Scientific Co., Pittsburgh, Pa.), washed five times with 2 ml of glass-distilled water, evaporated, and redissolved in 10 μl of chloroform. A 1-μl portion was injected into a Perkin-Elmer model 990 gas-liquid chromatograph equipped with dual flame ionization detectors and 2-m by 2-mm glass columns packed with 3% OV-225 on 100- to 120-mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. The injector and detector were kept at 235 and 250°C, respectively. The temperature was programmed to rise from 160 to 180°C at 1°C/min and from 180 to 200°C at 1.5°C/min. Arabinitol and mannose were well resolved from other sample components. The peaks were integrated with a System I Computing Integrator (Spectra-Physics, Santa Clara, Calif.). The relative response ratios of arabinitol and mannose to the internal standard were calculated, and quantitation was obtained by comparison with a standard curve. The standard curves for arabinitol and mannose were generated in triplicate by "spiking" samples of pooled normal human sera with doubling dilutions of both compounds from 100 to 0.2 μg/ml and a constant amount (7.5 μg) of the internal standard. The lowest sensitivity of this technique was 0.1 μg/ml, and the mean coefficient of variation within the concentration range from 0.39 to 50 μg/ml was 10.4%.

Double-antibody sandwich enzyme immunoassay for serum mannan was done as previously described (9). Briefly, mannan-serum complexes were dissociated by boiling for 3 min in an equal volume of 0.1 M disodium EDTA (pH 7.2). The gel was centrifuged, and the liquid phase (0.2 ml) was added to a polystyrene microtiter plate coated with a 1/5,000 dilution of anti-*C. albicans* cell wall immunoglobulin G (IgG). The plate was incubated for 1 h at 4°C and then washed. A 1/5,000 dilution of anti-*C. albicans* cell wall IgG conjugated to horseradish peroxidase was added (0.2 ml per well) and incubated for 1 h at 4°C. The substrate, *o*-phenylenediamine–H_2O_2, was then added, the color was developed for 1 h at 22°C, and the reaction was stopped with 25 μl of 4 M H_2SO_4. The absorbance at 490 nm of triplicate samples was compared with a standard curve for mannan spiked into serum at doses of 1 to 100 ng/ml. Data were expressed as the mean of the triplicates with respect to the standard curve.

The four immunosuppressed rabbits died of disseminated candidiasis on days 6, 7, 8, and 11, respectively, after infection. Cultural and histopathological evidence of disseminated candidiasis was obtained at autopsy. The kidneys yielded between 10^5 and 10^6 CFU of *C. albicans* cells per g, and the livers contained 10^2 to 10^4 CFU/g. In addition, the kidneys were enlarged, with multiple microabscesses. Mannanemia, negative before infection, peaked in all four rabbits 4 days after infection (mean, 32 ng/ml; range, 14.1 to 66.3 ng/ml) and decreased thereafter.

The chromatographic conditions resulted in good resolution of the regions corresponding to the retention times of arabinitol and mannose from other sample components. The internal standard, methyl α-D-mannopyranoside, did not interfere with the endogenous sample components and allowed reproducible quantitation of both metabolites. Marier et al. (4) were unable to resolve xylose, arabinitol, and ribitol as per-*O*-acetylated aldononitrile derivatives on a column packed with OV-225. We were also unable to separate these substances under similar conditions. However, Roboz et al. (10) showed by monitoring the ions having an *m/e* of 303 that the amount of ribitol and xylitol was less than 10% that of arabinitol in the majority of normal samples, as well as in those from infected patients. Furthermore, *Candida* species grown in human serum did not produce ribitol or xylitol.

Figure 1 shows the chromatograms of serial serum samples from a rabbit immunosuppressed with cortisone and infected with *C. albicans* 3181A. Serum concentrations of D-arabinitol and D-mannose were 0.26 and 16.9 μg/ml, respectively, 2 days before infection; 1.60 and 31.2 μg/ml, respectively, 4 days after infection; and 4.9 and 21.5 μg/ml, respectively, 8 days after infection. The range of serum concentrations of D-arabinitol and D-mannose in all four rabbits were, respectively, 0.25 to 0.32 and 12.1 to 16.9 μg/ml 2 days before infection, 0.49 to 0.79 and 21.5 to 26.4 μg/ml 2 days after infection, 0.44 to 1.6 and 12.9 to 31.2 μg/ml 4 days after infection, and 0.13 to 9.6 and 11.6 to 24.5 μg/ml 6 days after infection.

A 1,000-fold difference was observed between the mean peak mannan concentration (32 ng/ml) detected by sandwich enzyme immunoassay and the mean peak mannose concentration (25.9 μg/ml) assayed by GLC. Monson et al. (7) determined free mannose concentrations that were

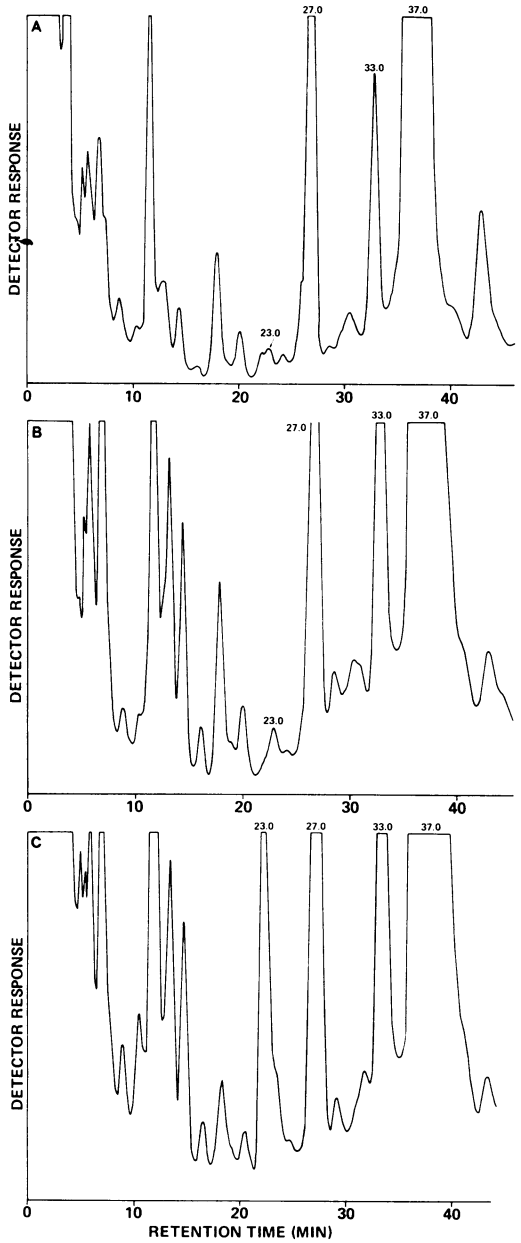

FIG. 1. Chromatograms of serial serum samples from a rabbit immunosuppressed with cortisone and infected with *C. albicans* 3181A; (A) 2 days before infection, (B) 4 days after infection, and (C) 8 days after infection. The peaks are: 23.0 min, arabinitol; 27.0 min, methyl α-D-mannopyranoside (internal standard); 33.0 min, mannose; and 37.0 min, glucose.

also in the microgram per milliliter range. Our observed serum mannan concentrations also agreed with those of Weiner et al. (13), who detected this substance in the nanogram per milliliter range. This 1,000-fold difference may be due to the following factors. (i) The amount of mannose measured represents the sum of endogenous mannose and mannose which is presumably derived from mannan. However, there was no endogenous mannan. (ii) A large part of the mannan may be metabolized into mannose soon after it is produced by *Candida* species. (iii) Circulating mannan can occur as soluble immune complexes which may decrease the sensitivity of existing assays, even with a dissociation step (9). (iv) Monson et al. (7) showed that *Candida* species can produce mannose in vitro.

We developed a model for disseminated candidiasis in immunosuppressed rabbits that allows the evaluation of tests that directly detect *Candida* species antigens or metabolites. A method was also devised for simultaneous quantitative determination of serum arabinitol and mannose levels by GLC. It will now be possible to compare these marker substances in this animal model.

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