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Short Communication

Diagnosis of disseminated candidiasis based on serum D/L-arabinitol ratios using negative chemical ionization mass spectrometry

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ABSTRACT

The main objective was to appraise the diagnostic specificity of the serum D/L-arabinitol ratio technique in a patient population biased for renal dysfunction. The D/L ratio (mean ± S.D.) in normal serum (n = 29) was 1.76 ± 0.47 (range 0.77–2.75). D/L-Arabinitol > 3.18 (mean + 3 S.D.) are considered indicative of disseminated candidiasis. Of 49 patients without candidiasis, but 46% with serum creatinine > 1.5 mg/dl, diagnostic specificity was 88%. In confirmed candidiasis (n = 16) sensitivity was 94% (D/L range 3.2–50.1). Switching from positive to negative chemical ionization permits the use of as little as 5 μl sample (20 μl used routinely; D/L ratios constant in the 5–200 μl range) permitting the extension of the technique to pediatric applications. Results can obtained in 2 h. Suggested areas of clinical application include aiding diagnosis, monitoring patients as risk so that treatment could be initiated while fungus load is still small, and following the course of antifungal chemotherapy.

INTRODUCTION

Disseminated candidiasis continues to be a frequent problem in the management of patients receiving cytotoxic, immunosuppressive, or corticosteroid therapy [1], with mortality rates in the 45–79% range [2]. Because there are no reliable non-invasive diagnostic procedures there has been a rapidly growing trend for empirical antifungal therapy (despite the considerable toxicities of the few available drugs) in neutropenic patients, adults as well as children, who remain persistently febrile under antibacterial chemotherapy [3–6].

Clinical diagnosis of disseminated candidiasis is difficult because there are no distinctive clinical manifestations [7], and histologic demonstration of tissue invasion, the only definitive method of diagnosis, is usually not practical. Blood cultures are not only slow (1.5 to 9 days) but detect candidemia in only less than one half of patients with disseminated candidiasis [8]. None of the many reviewed serodiagnostic techniques is adequate [2]. Current research efforts include *Candida* antigen latex tests [9] and circulating *Candida* enolase immunoassay [10]. Comparisons of various antibody, antigen, and metabolite techniques, and the commercial CAND-TEC assay were equiv-

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ocal and confirmed the need to improve diagnostic sensitivity as well as specificity [11,12].

The "arabinitol technique" is based on the fact that D-arabinitol, a pentitol, is a major metabolite of several pathologic *Candida* species. A recent review details available chromatographic and mass spectrometric techniques for the quantification of "arabinitol" (usually without separating the penitols and the arabinitol enantiomers) in serum and urine, discusses methodological shortcomings, and summarizes applications in human and experimentally infected animals [13].

Since its inception, a major weakness of the arabinitol technique has been the inability to differentiate between increased serum arabinitol concentrations due to renal dysfunction and *Candida* infection. This could be overcome by taking advantage of the fact that the important pathogenic *Candida* organisms produce only D-arabinitol [14,15]. There are two recent papers describing methodologies for the chiral chromatographic separation of the enantiomers of arabinitol in serum and urine. One technique uses multi-dimensional gas chromatography and advocates the reporting of results as arabinitol/creatinine ratios [16]. An alternative approach, from our laboratory, is to determine D/L-arabinitol ratios using direct chiral enantiomer separation with positive chemical ionization mass spectrometry and selected ion monitoring; this technique has the added benefit of eliminating the need for calibration analyses [17].

The primary objective of the present work was to appraise the diagnostic specificity (negativity when infection is not present) of the D/L-arabinitol ratio technique in a patient population biased for renal dysfunction. In addition, we switched from positive to negative chemical ionization, aiming to significantly increase analytical sensitivity, in anticipation of pediatric applications with very small sample quantities.

EXPERIMENTAL

The analytical technique employed was essentially the same as previously described in this journal [17], except for using negative instead of positive chemical ionization, which permitted the use of much smaller sample sizes, and several minor changes to improve derivatization, column life, and to accommodate the use of a different mass spectrometer system. Accordingly, just a general description of the methodology is given below and numerical and other details are provided only where experimental conditions were different from those given in ref. 17.

Sample preparation

Blood samples were collected in "red-top" glass tubes, and serum was obtained by centrifugation from clotted blood and stored frozen. To 20-μl serum aliquots (5–200 μl in the linearity experiments), 10 μl of internal standard (from a stock solution of 4.4 μg/μl concentration) were added and proteins were precipitated with acetone. After centrifuging, the supernatants were evaporated with nitrogen, the dried samples were dissolved in methylene chloride and derivatized with trifluoroacetic anhydride at 37°C for 40 min (instead of 75°C for 20 min as before). After evaporating the excess reagents, the residues were dissolved in 50 μl of toluene, and 1-μl aliquots were analyzed.

Gas chromatography–mass spectrometry

A gas chromatograph–single quadrupole mass spectrometer (Model 3300, Finnigan, San Jose, CA, USA), connected to an IBM AT computer with a Vector-One interface (Teknivent, Maryland Heights, MO, USA), was used. The analytical column (glass, coated with α-perpentylated cyclodextrin) was protected by a 1 m long uncoated, deactivated fused-silica capillary precolumn (Supelco, Bellefonte, PA, USA) which was connected to the metal on-column injector (Model 16715, Chrompack, Bridgewater, NJ, USA). A 1 m long bonded methylsilicone-coated capillary fused-silica column (Model 007-1-25W, Quadrex, New Haven, CO, USA) connected the analytical column to the ion source.

After on-column sample injection at room temperature, column temperature was increased from the initial 90–95°C at a rate of 1–2°C/min

until the last pentitol peak (xylitol) appeared (8–9 min), after which the temperature was lowered for the next injection. Helium was the chromatographic carrier gas, isobutane the chemical ionization reagent gas. The mass spectrometer was operated in the negative chemical ionization mode. Tuning parameters were adjusted to provide maximum intensity for the m/z 595 ions of tri-heptafluorobutylamine (FC-43), introduced through a variable inlet leak. Selected ion monitoring of m/z 518 and m/z 525 (internal standard) was carried out according to the protocols of the Vector-One software program.

D/L Ratio measurements and quantification

D/L-Arabinitol ratios were determined from peak areas. When needed, separate calibration curves were obtained for D- and L-arabinitol using normal serum samples supplemented with increasing quantities of the analytes.

Control and patient population

There were 29 control samples from normal blood bank donors. The population without evidence of disseminated candidiasis included 28 cancer patients with various hematological malignancies and solid tumors at assorted stages of progression, 20 patients with various renal dysfunctions, 1 patient with AIDS, and 1 post-surgical patient. There were 16 patients with blood culture and/or autopsy proven disseminated candidiasis.

RESULTS AND DISCUSSION

Methodology

The purpose of switching from positive to negative chemical ionization was to significantly reduce sample size requirement so that the technique could be extended to pediatric application. The high analytical sensitivity in negative chemical ionization results primarily from the high rate constants for electron capture which are due to the low mass and high mobility of electrons with respect to particle transfers [18], and also from the selective nature of ionization leading to reduced chemical noise by compounds other than the analyte. Arabinitol is not inherently electrophilic, however, the O-trifluoroacetyl derivative used provides substantial electron affinity.

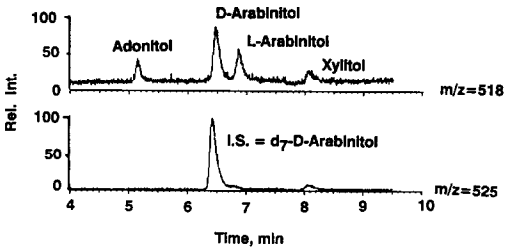


Fig. 1. Selected ion monitoring of the O-trifluoroacetylated derivatives of separated adonitol, xylitol and the enantiomers of arabinitol in a normal serum sample (20 μl sample size). I.S. = internal standard, fully deuterated D-arabinitol. Ions were obtained in negative chemical ionization using isobutane as reagent gas. Separation was accomplished using 20 m long glass capillary columns coated with α-perpentylated cyclodextrin; helium was used as carrier gas. The D/L ratio in this sample was 1.3.

The negative ion isobutane chemical ionization mass spectra of the O-trifluoroacetyl derivative of both D- and L-arabinitol (and also of adonitol and xylitol) were nearly identical. The $(M - 1)^-$ ion was at m/z 631 (approximately 12% intensity). The most intense (base) peaks at m/z 518, and m/z 525 for the internal standard, corresponding to the loss of an OCF_3CO group, were used for selected ion monitoring.

Fig. 1 shows the separation (to near baseline) and selected ion monitoring of D- and L-arabinitol, and also of adonitol (ribitol) and xylitol, in a 20-μl normal serum sample. The physical appearance of the tracing in Fig. 1 (and also of those in Fig. 3) is similar to those obtained in positive chemical ionization except that sample size used was ten times smaller than that needed with positive chemical ionization. Sample size linearity was checked by analyzing 5-, 10-, 20-, 30-, 40-, and 50-μl aliquots of a control serum sample. Individual D- and L-arabinitol concentrations increased linearly, with correlation coefficients of 0.93 and 0.96, respectively. The D/L ratios remained constant, 1.18 ± 0.35, regardless of sample size. Concerning analytical specificity, it is noted that no interference of any kind was en-

countered for either arabinitol enantiomer or the internal standard in hundreds of analyses.

The within-day injection reproducibility was determined by thirteen consecutive injections of a sample from a patient with confirmed candidiasis. The mean D/L-arabinitol ratio was 3.3 ± 0.4 with a coefficient of variation of 11%. Long-term reproducibility was determined by first analyzing six aliquots of a normal serum and than analyzing aliquots from the same sample eleven times during the next three months. The mean D/L-arabinitol ratios were 1.1 ± 0.1 and 1.2 ± 0.1 with the coefficients of variations of 11 and 9%, respectively. Comparable schedules using other samples gave coefficients of variation of similar magnitude.

A major advantage of using D/L-arabinitol ratios rather than the concentrations of the separated enantiomers is that there is no need for time-consuming calibration curves. There is no need for internal standard either, however, we still use one because its presence provides additional specificity for the D-arabinitol peak (only 7–8 s difference in retention times but at a different mass, see Fig. 1), its size reveals possible errors in sample preparation (although this does not effect the ratio measurements), and, if desired, a reasonably good (usually within 15%) quantification of the individual enantiomers may also be achieved by comparing their peak areas (or heights) with that of the internal standard, the concentration of which is known.

D/L-Arabinitol ratio in normal serum

In normal (blood bank) serum (n = 29), the mean ± S.D. of D/L-arabinitol ratio was 1.76 ± 0.47 (range 0.77–2.75). Because of the relatively narrow distribution of the D/L ratios in the normals (Fig. 2), the median, 1.7, was nearly the same as the mean. We defined the upper limit of normal as the mean + 3 S.D., thus D/L ratios >3.18 were considered abnormal and possible indicators of disseminated candidiasis.

Diagnostic sensitivity

Fig. 3 shows profiles, D/L-arabinitol ratios, total arabinitol concentrations (μg/ml), and creatinine values (mg/dl) for blood culture-confirmed candidiasis cases involving the two most common pathological candida species, *C. albicans* and *C. tropicalis*, and a case with *C. paratropicalis*, which is often difficult to diagnose. It is noted that despite the very high D/L ratio, creatinine was normal (<1.5 mg/dl) in the patient with *C. albicans* infection. The figure also illustrates a case of renal dysfunction but no candidiasis, where high creatinine and high total arabinitol were accompanied by a normal D/L ratio. This would have been a false positive by all techniques measuring only total arabinitol.

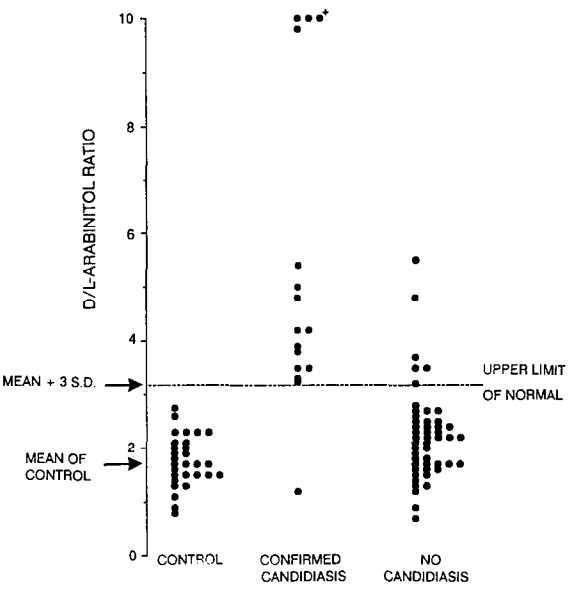
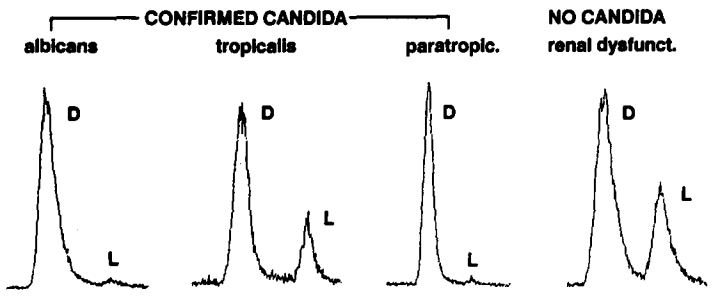


Fig. 2. Distribution of D/L-arabinitol ratios among controls (blood bank normals, n = 29), patients (n = 16) with confirmed disseminated candidiasis, and patients (n = 49) without candidiasis (population biased to include patients with renal dysfunction). The point marked by the + sign corresponds to a D/L ratio of 50.1.

Sixteen serum samples were analyzed from patients with confirmed candidiasis (73% had renal dysfunction). The D/L ratio was increased, >3.18 in fifteen cases, giving a diagnostic sensitivity of 94%. The one false negative, D/L = 1.2, was also negative using the positive chemical ionization technique. The D/L values ranged from 3.2 to 50.1; the distribution is shown in Fig. 2.



	albicans	tropicalis	paratropic.	renal dysfunct.
D/L	39.5	5.7	50.1	2.2
Creatinine, mg/dl	0.7	2.8	1.8	8.3
Arabinitol, μg/ml	4.3	1.2	23.9	2.5

Fig. 3. Monitoring of *m/z* 518 ions (obtained in negative chemical ionization with isobutane) of D- and L-arabinitol in serum samples. Experimental conditions were the same as in Fig. 1 (I.S. traces not shown here). Serum arabinitol D/L ratios >3.18 are considered abnormal and possible indications of disseminated candidiasis. The case of *C. albicans* illustrates that very high D/L ratios and increased total arabinitol may be accompanied by normal creatinine. The case of the renal dysfunction without candidiasis illustrates that serum D/L does not increase without candida infection even though the severe renal dysfunction results in increased total arabinitol. Creatinine >1.5 mg/dl and total arabinitol >0.8 μg/ml are considered increased.

Diagnostic specificity

Although endogenous arabinitol, the source of which is still unknown, contains both enantiomers, the fact that *Candida* species produce only D-arabinitol [14,16,17] can be utilized for the differential diagnosis of disseminated candidiasis based on changes in the D/L ratios [17]. Indeed, it was the main objective of this work to appraise the diagnostic specificity of the technique. Because the most common interference has been the presence of renal dysfunction, we have purposely selected a disproportionally large number of patients with renal insufficiency to assess potential interferences due to this condition.

Serum samples were analyzed from 49 patients, all without evidence of disseminated candidiasis but approximately one half (46%) with serum creatinine >1.5 mg/dl. The distribution of the D/L-arabinitol ratios is shown in Fig. 2. Forty-three samples yielded D/L-arabinitol ratios statistically indistinguishable (two-tailed *t*-test) from normals. There were six false positives, *i.e.*, D/L values >3.3. The diagnostic specificity was 88% for the population studied.

Of the six false positives, there were three cancer patients with infections, two with aspergillosis and one with oral candidiasis. These infections do not produce increased serum D/L ratios, however, aspergillosis patients often have concurrent candidiasis which can usually be confirmed only on autopsy. The other three false positives included a renal transplant patient and two patients (one cancer and one renal) without infection. The patient with oral candidiasis had normal creatinine, all others had increased creatinine (1.7–6.5 mg/dl). It is noted, for completeness, that one false positive was ignored; the sample was from an 88 year old renal patient, and we could not establish normal serum D/L-arabinitol for such an advanced age (we know that total endogenous arabinitol increases with advanced age).

The mean ± S.D. serum D/L-arabinitol ratios in a subpopulation of cancer patients without evidence of disseminated candidiasis, and with normal creatinine (n = 14), was 1.9 ± 0.5 (range 1.2–2.7); for seven patients with renal dysfunction (creatinine >1.5 mg/dl) the mean was 1.8 ± 0.3 (range 1.4–2.1). These values are statistically indistinguishable from the means obtained for normals (1.8 ± 0.5), confirming our previous observation [17,19] that neoplasms *per se* have

nothing to do with serum arabinitol, even in patients with concurrent renal dysfunction and/or advanced disease.

The diagnostic sensitivity of 94% (n = 16) and specificity of 88% (n = 49) of this technique are considered to be highly competitive to any method currently available for the diagnosis of disseminated candidiasis, particularly when one considers that sensitivity was assessed in a population selected to include about one half of the patients with renal dysfunction. In addition, there are several distinct methodological advantages including: as little as 5 μl serum is adequate (20 μl used routinely for adults), permitting pediatric applications; technical requirements are modest (most mass spectrometers have the required capabilities); the method is rather fast: when the instrument is set up, results can be reported in less than 2 h after receiving a sample.

Clinical applications are recommended in three areas: (1) individual sample analyses, when indicated, to support or confirm the diagnosis of disseminated candidiasis based on positive blood cultures and/or clinical observations; (2) serial, *e.g.*, weekly, monitoring of patients at risk for early diagnosis so that treatment could be initiated while the fungus load is still small; (3) relatively frequent, *e.g.*, twice a week, monitoring of patients undergoing antifungal chemotherapy to assist the evaluation of the efficacy of the treatment.

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