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Enantioselective Measurement of Fungal D-Arabinitol in the Sera of Normal Adults and Patients with Candidiasis

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A new method was used to measure D-arabinitol enantioselectively in the sera of 27 healthy adults and four patients with candidiasis. Arabinitol was measured by gas chromatography in serum that was treated with and without the *Klebsiella pneumoniae* enzyme D-arabinitol dehydrogenase, lactic dehydrogenase, NAD, and sodium pyruvate. Since enzyme treatment removed 98% of 0 to 20 μg of D-arabinitol per ml and none of 0 to 20 μg of L-arabinitol per ml from spiked sera, D-arabinitol could be determined from the difference in the treated and untreated samples. The concentrations of D- and L-arabinitol in serum from normal subjects were 0.22 ± 0.052 and 0.16 ± 0.055 μg/ml, respectively, and their D-arabinitol/creatinine and L-arabinitol/creatinine ratios were 0.024 ± 0.0089 and 0.017 ± 0.0053 (all means ± standard deviations). The infected patients all had markedly elevated serum D-arabinitol levels, but their L-arabinitol levels were either normal or proportionately much lower. The excess arabinitol in the sera of individuals with candidiasis is D-arabinitol, and use of enantioselective analytical methods should result in improved ability to diagnose and estimate the severity of candidiasis.

Traditional diagnostic methods such as blood cultures and serologic tests for antibodies fail to identify most immunocompromised patients with invasive candidiasis early enough for successful treatment. An alternative diagnostic approach is to demonstrate high concentrations of the *Candida* species metabolite D-arabinitol in body fluids. Previously studies have shown the following: (i) the species responsible for almost all human candidiasis produce large amounts of D-arabinitol in vitro (1, 10, 16, 22); (ii) animals and humans with invasive candidiasis have more arabinitol in their serum and urine than do uninfected controls (2, 3, 8, 10, 16, 17, 20–22); (iii) the arabinitol/creatinine concentration ratios in serum and urine are directly proportional to the rate at which arabinitol appears in the body from any source (21); (iv) the arabinitol/creatinine ratios in serum and urine of rats with experimental candidiasis are directly proportional to tissue colony counts (22); and (v) serum samples from most patients with invasive candidiasis have high arabinitol/creatinine ratios (8). Arabinitol is thus a quantitative diagnostic marker for candidiasis.

Arabinitol is also present in the serum and urine of individuals without candidiasis, however, and approximately one-third of infected patients have serum arabinitol/creatinine ratios that are within 2 standard deviations of the mean values in uninfected controls (8). We have proposed that it should be possible to differentiate fungal from nonfungal arabinitol (and thus improve diagnostic accuracy) on the basis of enantiomeric configuration. This is based on the fact that the medically important *Candida* species produce only D-arabinitol (2, 10), whereas known vertebrate metabolic pathways produce only L-arabinitol (19).

Two methods for measuring D-arabinitol enantioselectively have been described, but neither is satisfactory for analyzing serum. We therefore examined the abilities of several polyol dehydrogenase enzymes to deplete serum selectively of either D- or L-arabinitol. The *Klebsiella pneumoniae* enzyme D-arabinitol dehydrogenase (ADH) was most suitable and was used in combination with gas chromatography (GC) to measure D-arabinitol in serum. ADH converts D-arabinitol to D-xylulose by oxidizing the —OH group at position C-2 with concomitant reduction of NAD to NADH (23). The equilibrium of this reaction is unfavorable at physiologic pH levels, but ADH converts D-arabinitol to D-xylulose quantitatively when NAD is regenerated by a coupled reaction (11). Arabinitol was therefore measured by GC in two portions of serum, one of which was depleted of D-arabinitol with ADH, lactic dehydrogenase, NAD, and sodium pyruvate. Insofar as D-arabinitol is oxidized enantioselectively and nearly completely, its concentration can be determined from the difference in arabinitol concentrations in the enzyme-treated and untreated samples. This approach was then used to measure D- and L-arabinitol in the serum of 27 healthy adults and four patients with invasive candidiasis or *Candida* species fungemia.

MATERIALS AND METHODS

Enzyme assays. ADH and the related enzyme ribitol dehydrogenase (RDH) were assayed by measuring NADH generation spectrophotometrically (A_{340}) for 30 s at room temperature in a 1.0-cm curvette containing appropriately diluted enzyme, 0.83 μmol of NAD, and 50 μmol of either D-arabinitol or ribitol in 1.0 ml of 0.1 M phosphate buffer (pH 7.0) plus 10 mM 2-mercaptoethanol. One unit of enzyme activity was defined as the amount required to generate 1.0 μmol of NADH per min in this assay. D-Mannitol oxidation by ADH was assayed similarly.

Oxidation by ADH of D-arabinitol, L-arabinitol, or ribitol when NAD was regenerated by a coupled reaction was monitored by measuring the ketosugar reaction products by the cysteine-carbazole method (5) after the components described above, 0.5 mg sodium pyruvate, and 50 U of rabbit muscle lactic dehydrogenase (grade III; Sigma Chemical Co., St. Louis, Mo.; dialyzed against 0.1 M phosphate buffer [pH 6.0]–10 mM 2-mercaptoethanol before use) were held at 21°C for 60 min.

Preparation of purified ADH. *K. pneumoniae* DM516 (a

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uracil auxotroph that is a constitutive ADH overproducer) was shaken at 37°C for 24 h in liquid minimal salts medium (7) containing 0.001% uracil and 0.5% D-arabinitol and then for 48 h in minimal salts medium containing 0.001% uracil and 1% casein hydrolysate. A 25% suspension of washed cells was disrupted by sonication in 0.1 M potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol. The supernatants after centrifugation at 27,000 × *g* at 4°C for 30 min contained both ADH and RDH activity. Since RDH also catalyzes oxidation of L-arabinitol (6), ADH was purified by the method of Neuberger et al. (12), with minor modifications.

The following steps were conducted at 4°C in potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol (buffer) unless otherwise stated. The crude supernatant was diluted to 10 mg of protein per ml (Bio-Rad assay) in 0.1 M buffer, and nucleic acids were precipitated by adding 1 volume of 4% streptomycin sulfate per 10 volumes of supernatant. Solid ammonium sulfate was added slowly and with continuous stirring to the resulting supernatant, and the fraction that precipitated between 0.3 and 0.5 saturation was collected, dissolved in distilled water, and dialyzed against 0.01 M buffer. This fraction was further purified by ion-exchange chromatography (15- by 2.6-cm DEAE Sephacel column, elution with a 1,000-ml linear 0.01 to 0.15 M potassium phosphate gradient) and then by gel filtration (55- by 1.5-cm Ultrogel AcA44 column, elution with 0.1 M buffer). The active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 50% (vol/vol) glycerol in 0.02 M buffer–5 mM NAD. Samples were stored at −25°C and dialyzed overnight against 0.02 M buffer before use.

SDS-polyacrylamide gel electrophoresis. The crude bacterial sonicate and the active fractions after ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration were boiled for 3 min in 0.06 M Tris buffer (pH 6.8)–2% sodium dodecyl sulfate (SDS)–1% glycerol–5% 2-mercaptoethanol and examined by electrophoresis in a 0.1% SDS–12.5% polyacrylamide gel. The proteins were stained with Coomassie blue and with silver.

Enzyme treatment and derivatization. Three internal standards (50 μl of an aqueous solution of 80 μg each of xylitol, alpha-methylmannoside, and alpha-methylglucoside per ml) were added to two 0.2-ml portions of each serum sample. Then 0.8 U of ADH, 50 U of lactic dehydrogenase, 0.5 mg of sodium pyruvate, 0.5 mg of NAD, and 4.0 μg of D-mannitol were added to one of the samples. After thorough mixing, both samples were held at 21°C for 30 min, proteins were precipitated with 1.0 ml of acetone, and the supernatants after centrifugation (1,500 × *g*, 5 min) were evaporated to dryness in a nitrogen stream.

The trimethylsilyl ether derivatives were formed by adding 0.2 ml of a 1:1 mixture of trimethylsilylimidazole and *N*, *N*-dimethylformamide, sealing with Teflon, and heating to 50°C for 15 min. After the samples cooled to room temperature, the derivatives of interest were extracted into 0.25 ml of dry hexane.

GC. Two microliters of the upper hexane phase of each sample was analyzed with a Perkin Elmer Sigma 2000 gas chromatograph, a 0.32-mm by 60-m fused silica capillary GC column coated with a 1.0-μm film of SPB-5 (Supelco, Inc., Bellefonte, Pa.), helium carrier gas (flow rate, 40 cm/s at 195°C), and a flame ionization detector (275°C). Samples were introduced via a split-splitless capillary injector (2-mm-inner-diameter glass liner, 250°C) with the septum purge (5 ml/min) and inlet splitter (40 ml/min) closed for the first 30 s after injection. The oven temperature was 120°C for the first minute, and it was raised to 195°C at 25°C/min and then to 240°C at 3°C/min. Samples were analyzed for 22 min, and late-eluting components were removed by back flushing the column for an additional 13 min at 240°C.

The retention times of the compounds of interest were determined daily from a standard mixture; typical values were 14.18 min for xylitol, 14.45 min for arabinitol, 16.01 min for alpha-methylmannoside, 18.70 min for alpha-methylglucoside, and 20.22 min for mannitol.

Quantitation and controls. DL-Arabinitol (mixture of the D and L enantiomers) and mannitol were quantified from their relative peak areas compared with that of alpha-methylmannoside. Alpha-methylmannoside was used for quantitation because it was better separated from other sample components than was alpha-methylglucoside and because of variable recovery of xylitol from a few untreated serum samples.

Untreated and enzyme-treated portions of normal serum to which 20 μg each of D-arabinitol, D-mannitol, and the three internal standards per ml were added were analyzed daily to determine the detector response factors of the compounds of interest and the proportion of D-arabinitol that ADH removed from serum.

The mannitol that was added to each enzyme-treated sample was an additional internal control for the enzyme reaction. ADH also oxidizes D-mannitol, but more slowly than D-arabinitol (7, 12); disappearance of >95% of a large amount of D-mannitol thus implies that at least as much D-arabinitol also disappeared.

Samples studied. Completeness and enantioselectivity of D-arabinitol oxidation by ADH was assessed by measuring arabinitol in enzyme-treated and untreated normal human serum to which 20 μg of xylitol, 20 μg of alpha-methylmannoside, and either 0, 0.5, 5, or 20 μg of D- or L-arabinitol per ml had previously been added.

The accuracy and precision of the DL- and D-arabinitol measurements were assessed by analyzing on 4 different days enzyme-treated and untreated normal serum to which 0, 0.2, 2, 10, or 20 μg of D-arabinitol per ml had been added.

Arabinitol was then measured in enzyme-treated and untreated portions of single serum specimens from 27 healthy adults and in nine serum samples from four patients with either histologically proven invasive candidiasis or fungemia due to *Candida albicans* or *C. tropicalis*. The infected patients were previously known to have high serum DL-arabinitol levels. Creatinine was measured with an autoanalyzer, and the arabinitol results were corrected for effects of renal function by calculating the arabinitol/creatinine ratios as previously described (8, 21).

RESULTS

Characterization of ADH. The crude *K. pneumoniae* DM516 sonic extract contained 0.92 U of ADH and 1.41 U of RDH per mg of protein, and the final enzyme preparation contained 11.6 U of ADH and no measurable RDH (<0.0005 U) per mg of protein. The overall yield of ADH was 53%. Purified ADH oxidized D-arabinitol 1.5 times faster than D-mannitol, and it oxidized no measurable ribitol or L-arabinitol in the presence of excess lactic dehydrogenase and sodium pyruvate. SDS-polyacrylamide gel electrophoresis showed that the final product contained a few faint bands representing proteins other than ADH (molecular weight, 46,000 [12]), but none of these corresponded to RDH (mol wt = 108,000) or its subunits (molecular weight, 27,000 [18]) (Fig. 1). The final preparation retained 80% of its original ADH activity after storage at −25°C for 4 months.

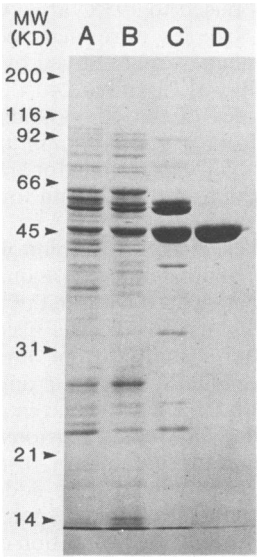


FIG. 1. Fractions obtained during purification of ADH were denatured and examined by SDS-polyacrylamide gel electrophoresis. A few faint bands other than ADH (46 kilodaltons) were found in the final product, but none of these corresponded to RDH (108 kilodaltons) or its 27-kilodalton subunits. Lanes: A, cell-free supernatant of sonicated *K. pneumoniae* DM516; B, 0.3 to 0.5 saturation ammonium sulfate fraction; C, active fractions from DEAE-Sephacel column; D, final preparation after gel filtration (30 μg of protein per lane, Coomassie blue stain). Molecular sizes in kilodaltons are indicated to the left of the gels.

Enzyme activity in serum. The enzyme-treated normal sera to which the internal standards and 0 to 20 μg of D-arabinitol per ml had previously been added contained 0.020 ± 0.001 times as much arabinitol as the untreated samples (mean ± standard deviation of the ratio of the slopes of the linear regression lines relating the amounts recovered to the known amounts added; four experiments). In contrast, the enzyme-treated samples to which 0 to 20 μg of L-arabinitol per ml had been added contained 0.99 ± 0.055 times as much arabinitol as the untreated samples. Since these results showed that enzyme treatment removed 98% of D-arabinitol and no measurable L-arabinitol from serum, serum D- and L-arabinitol concentrations (concentration indicated by brackets) were subsequently determined as follows: [D-arabinitol] = ([arabinitol]$_{\text{no enzyme}}$ − [arabinitol]$_{\text{enzyme}}$)/(proportion D-arabinitol removed from control) and [L-arabinitol] = [arabinitol]$_{\text{no enzyme}}$ − [D-arabinitol].

Accuracy and precision. The results of four replicate analyses on different days of sera containing 0.2 to 20.2 μg of D-arabinitol per ml are shown in Table 1. The mean measured DL- and D-arabinitol concentrations were within either 0.03 μg/ml or 4% of the expected amounts (amount added plus mean *y* intercept of the linear regression lines in four experiments), and the standard deviations were no more than 0.1 μg/ml or 9%.

Results in normal subjects and patients with candidiasis. The serum D- and L-arabinitol results in the normal subjects and the patients with candidiasis are shown in Table 2, and chromatograms of enzyme-treated and untreated serum from an infected patient are shown in Fig. 2. The D- and L-arabinitol concentrations in sera from normal subjects were 0.22 ± 0.052 and 0.16 ± 0.055 μg/ml, respectively and their D-arabinitol/creatinine and L-arabinitol/creatinine ratios were 0.024 ± 0.0089 and 0.017 ± 0.0053, respectively (all means ± standard deviations). The infected patients all had markedly elevated D-arabinitol levels, but their L-arabinitol levels were either normal or comparatively much lower. D-arabinitol accounted for 88 ± 9% of total arabinitol in the final serum specimens from the four infected patients compared with 60 ± 9% (mean ± standard deviation) in the sera from normal patients ($P < 0.0001$ by Student's *t* test).

DISCUSSION

Two methods for measuring D-arabinitol enantioselectively have previously been described, but technical problems limit the usefulness of these methods for analysis of serum. Bernard et al. (2) measured arabinitol by GC in samples that were incubated for 24 h with a strain of *C. tropicalis* that consumed D-arabinitol once preferred substrates were exhausted; the difference in arabinitol concentration between this sample and another that was not treated microbiologically was considered to be the D-arabinitol concentration. There were several problems with this approach. First, *C. tropicalis* consumed D-arabinitol incompletely in samples that contained antifungal drugs. Second, the GC method was not sensitive enough to quantify the small amounts of arabinitol in most sera that survive microbiologic treatment. Last, results could not be obtained rapidly because of the 24-h incubation step. Despite these limitations, this study established that D-arabinitol is responsible for most of the increases in body fluid and tissue arabinitol levels in candidiasis.

Soyama and Ono (17) used a different approach. D-Arabinitol concentrations were derived from the initial rates of NADH generation when NAD and partially purified *K. pneumoniae* ADH were added to serum extracts. This is an attractive approach because it does not include a time-consuming chromatography step. However, ADH also oxidizes D-mannitol (7, 12), and D-mannitol generated NADH

TABLE 1. Accuracy and precision of serum DL- and D-arabinitol measurements

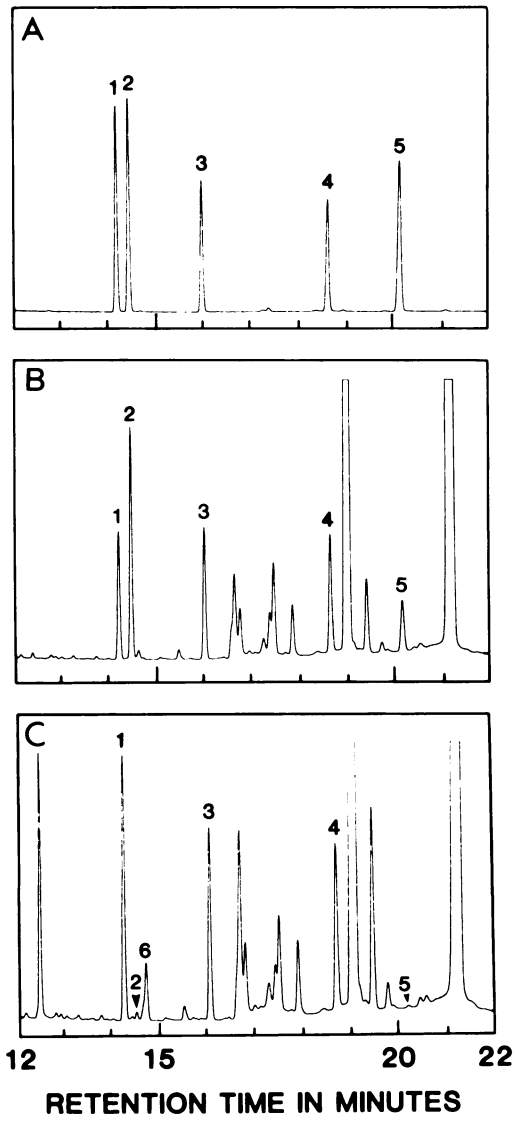
D-Arabinitol added (μg/ml)	DL-Arabinitol concn (μg/ml)				D-Arabinitol concn (μg/ml)			
	Expected[a]	Observed[b]	Obs/exp ratio[c]	CV[d] (%)	Expected	Observed	Obs/exp ratio	CV (%)
0	0.43	0.40 ± 0.078	0.93	20	0.22	0.20 ± 0.033	0.89	17
0.2	0.63	0.63 ± 0.10	1.00	16	0.42	0.42 ± 0.044	0.99	11
2.0	2.43	2.52 ± 0.16	1.04	6.3	2.22	2.31 ± 0.18	1.04	7.7
10.0	10.4	10.4 ± 0.88	1.00	8.4	10.2	10.2 ± 0.91	1.00	8.9
20.0	20.4	20.6 ± 1.5	1.01	7.2	20.2	20.4 ± 1.4	1.01	7.0

[a] Amount added plus mean *y* + intercept (n = 4 experiments).
[b] Mean ± standard deviation on 4 different days.
[c] Ratio of observed results to expected results.
[d] CV, Coefficient of variation.

TABLE 2. D- and L-arabinitol levels in serum samples from normal adults and patients with candidiasis

Serum source (date)	Concn (μg/ml)			Ratio	
	D-Arabinitol	L-Arabinitol	Creatinine	D-Arabinitol/ creatinine	L-Arabinitol/ creatinine
Normal adults	0.22 ± 0.052[a]	0.16 ± 0.055[a]	0.95 ± 0.20[a]	0.024 ± 0.0089[a]	0.017 ± 0.0053[a]
Candidiasis patients					
1	4.3	1.1	3.8	0.11	0.029
2	5.0	1.5	3.2	0.16	0.047
3 (29 April)	13	0.19	1.5	0.87	0.013
3 (30 April)	25	0.36	1.8	1.3	0.020
4 (18 January)	0.17	0.19	0.8	0.021	0.024
4 (22 January)	0.30	0.16	0.9	0.033	0.018
4 (24 January)	0.21	0.18	0.9	0.023	0.020
4 (27 January)	5.5	0.37	1.7	0.30	0.022
4 (28 January)	45	2.1	1.9	2.4	0.11

[a] Mean ± standard deviation (n = 27).



20% as efficiently as D-arabinitol in this assay. Since normal serum contains approximately equimolar amounts of D-mannitol and DL-arabinitol (15), D-arabinitol concentrations determined by this technique can be expected to be at least 20% too high. This lack of substrate specificity and possible prescence of other NAD oxidoreductases in the partially purified ADH preparation probably explain why the authors found more D-arabinitol in normal human sera (mean, 1.0 μg/ml; n = 42) than the amounts of DL-arabinitol reported by several other laboratories (2, 3, 8, 10, 13–16, 20, 21).

Our new method also uses bacterial ADH, but problems due to the lack of absolute substrate specificity of ADH were avoided by using GC for quantitation. Enzymatic depletion of D-arabinitol from specimens is preferable to the microbiologic method because antimicrobial agents do not interfere and because the new method is much easier and faster. Some time and effort are required to purify and characterize ADH, but this enzyme is sufficiently stable so that many months' supply can be prepared at once.

It was also necessary to modify the former GC method for measuring arabinitol (21). Sensitivity was improved by using a capillary column instead of a packed column to obtain taller and narrower peaks and by analyzing large amounts of the hexane extracts of the derivatized samples without inlet splitting. We also found that hexamethyldisilazane and trimethylchlorosilane could not be used to derivatize enzyme-treated sera; the unstable trimethylsilyl enol of pyruvate produced multiple interfering peaks. Enolization of pyruvate did not occur when trimethylsilylimidazole was used. With these modifications, arabinitol was measurable in all of the enzyme-treated and untreated sera; the lowest arabinitol concentration in any sample was 0.08 μg/ml.

FIG. 2. GC analysis (12 to 22 min only) of (A) a solution of equal amounts of xylitol (peak 1), D-arabinitol (peak 2), alpha-methylmannoside (peak 3), alpha-methylglucoside (peak 4) and D-mannitol (peak 5) and of untreated (B) and ADH-treated (C) serum from candidiasis patient 3. This serum contained 25 μg of DL-arabinitol per ml, 98% of which was converted by ADH to D-xylulose (peak 6) and was thus D-arabinitol. Analytical conditions: 0.32-mm by 60-m fused silica SPB-5 GC column (1.0-μm film); helium carrier at 40 cm/s; splitless injection (no solvent effect); flame ionization detector; column temperature at 120°C for 1 min with an increase to 195°C at 25°C/min and then to 240°C at 3°C/min.

It was thus possible to define the ranges of serum D- and L-arabinitol levels in normal adults. We found that the normal subjects had higher DL-arabinitol/creatinine and D-arabinitol/creatinine ratios and higher proportions of D-arabinitol relative to DL-arabinitol in their sera than were found earlier in 10 normal adult urine samples (2). The normal subjects also had lower serum DL-arabinitol/creatinine ratios than an earlier group of 88 uninfected patients with cancer or renal failure (8). These differences may have been due to the different methods used to deplete samples of D-arabinitol and to measure arabinitol by GC. It is also possible that the proportions of D- and L-arabinitol are not the same in serum and urine and that hospitalized cancer patients who do not have candidiasis indeed have more D- or L-arabinitol in their sera than normal persons. The availability of a sensitive and accurate enantioselective method for measuring D-arabinitol should facilitate future studies of the origins of D- and L-arabinitol in body fluids, the means by which D- and L-arabinitol are eliminated from the body, and whether renal failure, corticosteroid administration (4), sarcoidosis (9), and microbial colonization influence body fluid D- or L-arabinitol levels.

The present study has shown that normal human serum contains both D- and L-arabinitol but that D-arabinitol is the enantiomer responsible for the high serum arabinitol levels in patients with candidiasis. All four infected patients had high serum D-arabinitol/creatinine ratios, but their L-arabinitol/creatinine ratios were either normal or comparatively much lower. The serial results from patient number 4 further illustrate this point. This patient's first three serum samples contained normal amounts of DL-arabinitol, and 47, 65, and 54%, respectively, of this arabinitol was the D-enantiomer. In contrast, the last two serum samples contained large amounts of DL-arabinitol, and 93 and 95%, respectively, of this arabinitol was the D-enantiomer. These results confirm those of Bernard et al. (2), and they support our hypotheses that (i) excess arabinitol in candidiasis is of fungal rather than host origin and (ii) the diagnostic accuracy of arabinitol measurements in candidiasis can be improved by using enantioselective analytical methods.

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