A Rapid, Automated Enzymatic Fluorometric Assay for Determination of d-Arabinitol in Serum

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Received 20 September 1999/Returned for modification 23 December 1999/Accepted 2 February 2000

A rapid enzymatic fluorometric assay for measuring d-arabinitol in serum was developed using recombinant d-arabinitol dehydrogenase from Candida albicans (rArDH). rArDH was produced in Escherichia coli and purified by dye-ligand affinity chromatography. rArDH was highly specific for d-arabinitol, cross-reacting only with xylitol (4.9%) among all polyols tested. A Cobas Farara II centrifugal autoanalyzer (Roche) was used to measure NADH fluorometrically when rArDH and NAD were added to serum extracts, and d-arabinitol concentrations were calculated from standard curves derived from pooled human serum containing known amounts of d-arabinitol. The method was precise (mean intra-assay coefficients of variation [CVs], 0.8%), and mean interassay CVs, 1.6% and rapid (3.5 min per assay) and showed excellent recovery of added d-arabinitol in serum (mean recovery rate, 101%). The mean and median d-arabinitol/creatinine ratios were 2.74 and 2.23 μM/mg/dl, respectively, for the 11 patients with candidemia compared to 1.14 and 1.23 μM/mg/dl, respectively, for 10 healthy controls (P < 0.01). These results confirm earlier studies showing that serum d-arabinitol measurement may help to promptly diagnose invasive candidiasis. The technique shows a significant improvement in terms of accuracy, cost, simplicity, specificity, and speed compared with gas chromatography, mass spectrometry, and earlier enzymatic assays.

As the incidence of invasive candidiasis has increased dramatically in recent years, the accurate and early detection of this infection has become of major importance. Unfortunately, conventional culture-based clinical methods, which may take several days to become positive, are not very sensitive for detecting invasive disease (1, 7, 14, 15). Alternative approaches such as PCR assays and immunodiagnostic methods have been described, but these methods are not yet sufficiently sensitive and specific to have been widely adopted in clinical practice (15).

D-Arabinitol is a metabolite of several pathogenic Candida species, and several studies have shown that serum d-arabinitol concentrations and serum d-arabinitol/creatinine ratios are higher in humans and animals with invasive candidiasis than in uninfected or colonized controls (4, 9, 18, 19). Early studies used gas chromatography (GC) or GC-mass spectrometry to detect and quantify d-arabinitol in serum (2, 4, 5, 9). However, these methods require expensive equipment, and specimen processing and analysis require considerable time and effort. Enzymatic assays that use d-arabinitol dehydrogenase from Klebsiella pneumoniae (10, 11) to quantify d-arabinitol are less cumbersome, but these enzymes also react with D-mannitol, which is sometimes present in human serum (8). In 1994, Switchenko et al. (13) described a colorimetric endpoint enzymatic assay that used a clinical chemistry autoanalyzer and d-arabinitol dehydrogenase (ArDH) from Candida tropicalis to quantify d-arabinitol in serum (13). The ArDH utilized in this study was extracted and purified from C. tropicalis. A method for overproducing recombinant C. tropicalis ArDH in Escherichia coli has since been described (6), but recombinant ArDH (rArDH) has not yet been used in automated d-arabinitol assays.

In this study, we describe a sensitive, specific, and rapid enzymatic fluorometric method for measuring d-arabinitol in serum utilizing an automated analyzer. This new assay is faster and simpler than methods employing GC, GC-mass spectrometry, or the colorimetric endpoint enzymatic assay. Moreover, fewer reagents are required, and the automated equipment is available in many clinical laboratories. The key reagent is rArDH from Candida albicans, which was overproduced in E. coli and purified to homogeneity by dye-ligand affinity chromatography. The assay is based on oxidation of d-arabinitol to d-ribulose by rArDH, with the concomitant reduction of NAD to NADH. The initial rate of NADH production, which is proportional to the amount of d-arabinitol in serum, is measured fluorometrically. The d-arabinitol concentration was determined by comparing the initial rate of NADH production to that for d-arabinitol calibration curves.

**MATERIALS AND METHODS**

**Expression, purification, and properties of rArDH.** (i) Production and purification of rArDH. In order to produce recombinant C. albicans ArDH (rArDH) in E. coli, we used PCR to amplify the C. albicans ARDI coding sequence from plasmid pB4 (20) and to change the CATAATGAT sequence at the start codon (underlined) to CACCATGGAT, thereby introducing an NcoI restriction site. The PCR product was digested with NcoI and XbaI and ligated into NcoI and XbaI-digested pET19b (Novagen), which yielded pET19b/ArDH. Next, the portion of the ARDI coding sequence 3′ to the SpeI restriction site was excised from pET19b/ArDH with SpeI and XbaI and replaced with the SpeI-XbaI fragment from pBl4, which yielded pET19b/ArDH1. Lastly, all of the ARDI coding sequence 5′ to the SpeI restriction site in pET19b/ArDH1 was sequenced to verify that no errors had been introduced by PCR.

Next, E. coli BL21(D3) (Novagen) was transformed with pET19b/ArDH1, and the transformants were grown to an optical density value at 600 nm of 0.6 in Circlegrow broth (Bio 101, Vista, Calif.) supplemented with 50 μg of ampicillin (Sigma, St. Louis, Mo.) per ml at 37°C with shaking. Isopropyl-β-D-thiogalactopyranoside (American Bioanalytical, Natick, Mass.) was added to 1 mM, and the cells were shaken at 37°C for 3 more h. The cells were harvested by centrifuga-

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tion and suspended in 100 mM sodium phosphate buffer (pH 7.0) (2 ml/100-ml cultures). The cells were broken by sonication for four periods of 10 s each, and cellular debris was pelleted by centrifugation at 30,000 g for 30 min. The supernatant was cleared by ultracentrifugation at 100,000 g for 45 min, and it was loaded onto a reactive Yellow 86 column (2.5 by 20 cm) (Sigma) and washed with 350 ml of 100 mM sodium phosphate buffer (pH 7.0) and with 350 ml of 100 mM sodium phosphate buffer (pH 7.0) plus 0.5 M NaCl. rArDH was eluted with 50 ml sodium phosphate buffer (pH 7.0)–200 mM NaCl–5 mM MgSO4–1 mM NADH. Active fractions were pooled and concentrated by ultrafiltration (Centricon-30; Amicon, Danvers, Mass.). NADH was removed with a desalting column (Econo-Pac DG; Bio-Rad, Richmond, Calif.), and the purified rArDH was stored at −80°C in 0.1 M sodium phosphate buffer (pH 7.0)–200 mM NaCl–5 mM MgSO4.

(ii) Characterization of rArDH. The purity of rArDH was assessed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a 12% polyacrylamide gel. rArDH catalytic activity was monitored using a UV-2001 PC Spectrophotometer (Shimadzu Scientific, Inc., Braintree, Mass.). The 1-ml reaction mixture contained 50 mM glycine (pH 9.5), 0.1 U of rArDH, and 10 μl of 50 mM NAD; the reaction was initiated by the addition of 50 μl of 15% (wt/vol) D-arabinitol. Specific activity is reported as units per milligram of protein, where 1 U is defined as the amount required to generate 1 μmol of NADH/min. For substrate and cofactor specificity studies, the reaction mixture contained 50 mM reagent containing 50 mM NaCl, 0.1 U of enzyme, and 10 μl of 50 mM substrate. The reverse reaction was assayed in 50 mM glycine (pH 7.0), containing 0.34 mM NADH, 0.1 U of rArDH, and 100 mM substrates.

To test the effect of pH on the enzyme activity, buffers containing 50 mM sodium citrate, 50 mM sodium phosphate, and 100 mM Tris with pH values ranging from 4.0 to 10.5 were used.

Fluorometric enzymatic assay for measuring serum d-arabinitol concentration. (i) Serum treatment and analysis. Serum was diluted 1:1 (vol/vol) with 100 mM sodium citrate (pH 4.0), boiled for 10 min, cooled on ice, and then centrifuged for 10,000 g for 10 min. The supernatant was analyzed immediately or stored at −20°C for later analysis.

Six calibrators were prepared by supplementing a normal pooled human serum with different concentrations of D-arabinitol. These included acetaminophen (300 μg/ml), gentamicin (120 μg/ml), amphotericin B (20 μg/ml), itraconazole (16 μg/ml), ketoconazole (16 μg/ml), cefacor (230 μg/ml), ciprofloxacin (43 μg/ml), erythromycin (200 μg/ml), gentamicin (120 μg/ml), chloramphenicol (250 μg/ml), vancomycin (630 μg/ml), and heparin (8 U/ml). None of these drugs produced a measurable response. Serum d-arabinitol concentrations and d-arabinitol/creatinine ratios in patients with candidemia. Serum d-arabinitol concentration and suspended in 100 mM sodium phosphate buffer (pH 7.0) (2 ml/100-ml cultures). The cells were broken by sonication for four periods of 10 s each, and cellular debris was pelleted by centrifugation at 30,000 g for 30 min. The supernatant was cleared by ultracentrifugation at 100,000 g for 45 min, and it was loaded onto a reactive Yellow 86 column (2.5 by 20 cm) (Sigma) and washed with 350 ml of 100 mM sodium phosphate buffer (pH 7.0) and with 350 ml of 100 mM sodium phosphate buffer (pH 7.0) plus 0.5 M NaCl. rArDH was eluted with 50 ml sodium phosphate buffer (pH 7.0)–200 mM NaCl–5 mM MgSO4–1 mM NADH. Active fractions were pooled and concentrated by ultrafiltration (Centricon-30; Amicon, Danvers, Mass.). NADH was removed with a desalting column (Econo-Pac DG; Bio-Rad, Richmond, Calif.), and the purified rArDH was stored at −80°C in 0.1 M sodium phosphate buffer (pH 7.0)–200 mM NaCl–5 mM MgSO4.

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centrations ranged from 1.10 to 1.58 μM in 10 healthy controls and 1.64 to 19.11 μM in the 11 patients with Candida fungemia. The mean and median D-arabinitol/creatinine ratios were 1.14 and 1.23 μM/mg/dl, respectively, for the healthy subjects, and they were 2.74 and 2.23 μM/mg/dl, respectively, for the patients with candidemia (Fig. 3). The infected patients’ D-arabinitol/creatinine ratios were significantly higher than the values for the healthy subjects (P < 0.01 by the Mann-Whitney test).

DISCUSSION

Many medically important species of Candida (including C. albicans, C. tropicalis, and C. parapsilosis) produce measurable amounts of D-arabinitol in culture as well as in infected animals and humans (3, 4, 5, 18). It has been shown elsewhere that patients with invasive candidiasis had higher serum D-arabinitol levels than did uninfected controls and that these levels declined with effective therapy (2, 4, 19). Based on these results, D-arabinitol has been recognized as a potentially useful diagnostic marker for invasive candidiasis. Since D-arabinitol is cleared primarily by glomerular filtration, the level of D-arabinitol in serum increases in proportion to creatinine when renal function is impaired. To correct for this effect, we and

FIG. 1. Calibration curve for the assay of D-arabinitol in spiked human serum. A pooled human serum containing 1.1 μM endogenous D-arabinitol was supplemented with six different concentrations of D-arabinitol. The x axis gives the total concentrations of endogenous plus added D-arabinitol, whereas the y axis gives the mean ± SD of initial rates of change in fluorescence per minute assayed on six different days (R = 0.9995).

FIG. 2. Accuracy of D-arabinitol measurement determined by automated fluorometric assay. Eight serum samples that were spiked with different concentrations of D-arabinitol were assayed by the automated fluorometric method, and D-arabinitol values are shown on the y axis (mean ± SD). The D-arabinitol concentration on the x axis is predicted from the total concentrations of endogenous and added D-arabinitol (R = 0.9990).

TABLE 1. Recoveries of 2 μM D-arabinitol following its addition to nine healthy sera

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Measured D-arabinitol concn (μM)</th>
<th>% Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unsupplemented sample</td>
<td>Sample supplemented with 2 μM D-arabinitol</td>
</tr>
<tr>
<td>1</td>
<td>1.25 ± 0.06</td>
<td>3.26 ± 0.14</td>
</tr>
<tr>
<td>2</td>
<td>1.20 ± 0.01</td>
<td>3.20 ± 0.10</td>
</tr>
<tr>
<td>3</td>
<td>1.44 ± 0.02</td>
<td>3.48 ± 0.17</td>
</tr>
<tr>
<td>4</td>
<td>1.42 ± 0.06</td>
<td>3.38 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>1.41 ± 0.03</td>
<td>3.35 ± 0.34</td>
</tr>
<tr>
<td>6</td>
<td>1.20 ± 0.14</td>
<td>3.11 ± 0.18</td>
</tr>
<tr>
<td>7</td>
<td>1.22 ± 0.01</td>
<td>3.21 ± 0.11</td>
</tr>
<tr>
<td>8</td>
<td>1.24 ± 0.10</td>
<td>3.34 ± 0.10</td>
</tr>
<tr>
<td>9</td>
<td>1.19 ± 0.04</td>
<td>3.31 ± 0.10</td>
</tr>
</tbody>
</table>

* Difference between measured supplemented and unsupplemented D-arabinitol concentration divided by D-arabinitol concentration added.
steady source of rArDH, we cloned the C. albicans
C. tropicalis from D-arabinitol in human serum using a highly specific ArDH to measure D-arabinitol. However, these methods are compli-
cated due to the unavailability of rapid and simple analytical
marker, its routine use in the clinical laboratory has been
others have used serum D-arabinitol/creatinine ratios (4, 16, 17,
Despite the usefulness of D-arabinitol as a diagnostic
marker, its routine use in the clinical laboratory has been
limited due to the unavailability of rapid and simple analytical
methods. GC and GC-mass spectrometry were used originally
to measure D-arabinitol. However, these methods are compli-
cated, technically demanding, and time-consuming and may
allow only a small number of samples to be tested each day (8,
9). The necessary equipment is also very expensive and is not
available in hospital diagnostic laboratories. Hence, an enzymatic
test that used ArDH from K. pneumoniae was developed (10, 11, 12).
However, this enzyme showed 20% cross-reactivity with D-mannitol, a sugar that may be present in
Thus a method for overproducing recombinant C. tropicalis ArDH has been described previously (6), rArDH has not yet
been used in an automated D-arabinitol assay. To ensure a
steady source of rArDH, we cloned the C. albicans structural
gene, overproduced rArDH in E. coli, and purified rArDH to homogeneity by dye-ligand affinity chromatography. Thus, it is
now possible to generate a highly purified rArDH that is highly
specific for D-arabinitol and has no cross-reactivity with sugars
commonly found in human serum such as L-arabinitol, mannito,
and sorbitol. With a readily obtainable recombinant en-
zyme, it is technically feasible to detect and quantify D-arabini-
tol using clinical chemistry equipment.

The automated fluorometric enzymatic assay described here
is much faster and simpler to use than either GC or GC-mass
spectrometry. The assay has been set to perform automatically
on a Cobas Fara II centrifugal autoanalyzer, thereby simplifying
the procedure. The automated enzymatic D-arabinitol assay
developed by Switchenko et al. (13) was based on two reac-
tions; the first reaction is the oxidation of D-arabinitol and the
concomitant reduction of NAD to NADH. In the second re-
aaction, the NADH reduces p-iodonitrotetrazolium violet to
iodonitrotetrazolium-formazan, which is measured spectro-
photometrically. In the fluorometric assay described here, the
coupling reagent and dye reagent are eliminated, saving both
time and cost in reagent preparation and reducing potential
sources of error. Our reaction system, which is based on initial
reaction rate, is approximately five times faster than the dye-
coupling method using the endpoint reaction. The dye-cou-
pling method requires 16 min for one assay, whereas the flu-
orometric assay requires only 3.5 min, thereby increasing
sample throughput substantially.

We have used the enzymatic fluorometric assay to monitor
the levels of D-arabinitol in the serum of healthy controls and
patients with candidemia. Although the numbers of studied
samples were small, significantly elevated D-arabinitol/creati-
nine ratios were found in the serum from patients with invasive
candidiasis compared to the ratios for uninfected controls.
These data, in conjunction with other clinical indicators, may
provide earlier detection of invasive candidiasis, which in turn
may facilitate the earlier treatment of invasive candidiasis.
The enzymatic fluorometric assay that we developed is sim-
ple, highly specific, and sensitive for measuring D-arabinitol in
serum and permits analysis of many samples within a working
day. Currently, we have an ongoing prospective project in the
State of Connecticut for retrieving serum specimens from pa-
tients diagnosed with candidemia. The study will allow us to
explore the potential role of monitoring serum D-arabinitol
concentration by the fluorometric method described here. Al-
though blood culture is unlikely to be replaced by other diag-
nostic tools, we anticipate that fluorometric detection of D-
arabinitol will ultimately lead to improved diagnosis of invasive
candidiasis.

ACKNOWLEDGMENTS

This work was supported by grants from the U.S. Department of Veterans’ Affairs and from Pfizer Pharmaceuticals, Inc.

REFERENCES

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