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SEPARATION AND QUANTIFICATION BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF ARABINITOL ENANTIOMERS TO AID THE DIFFERENTIAL DIAGNOSIS OF DISSEMINATED CANDIDIASIS

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SUMMARY

To differentiate increased arabinitol due to fungal (only D-arabinitol) and non-fungal origin, O-trifluoroacetyl derivatives of the enantiomers were separated using α-perpentylated cyclodextrin columns and measured by selected ion monitoring. Mean ± S.D. D/L in normal serum: 1.40 ± 0.42. D/L ratios >2.24, defined as normal mean + 2S.D., were considered outside normal range. D/L was >2.2 in 10 of 12 confirmed candidiasis cases with one false negative and one borderline. Renal dysfunction without candidiasis yielded normal D/L despite high arabinitol concentrations. D/L in normal urine was nearly identical to that in serum despite 60 times larger concentration. D/L ratios, determined by peak heights or areas, could be used without the need to determine concentrations.

INTRODUCTION

Improvements in the management of bacterial infections led to an increased frequency of systemic fungal infections among neutropenic cancer patients, particularly among those with hematological neoplasms[1,2]. Fungal infections also complicate renal transplantation, parenteral nutrition and other indwelling catheters, the management of high risk neonates and burn patients, drug abusers and patients with the acquired immunodeficiency syndrome (AIDS). *Candida* species are the most frequent cause of fungal infections in patients receiving high doses of cytotoxic, immunosuppressive, or corticosteroid therapy. Because disseminated candidiasis (called candidosis in Europe) is a life-threatening infection which occur with increasing frequency, and because treatment is often inadequate, there is a considerable current interest in all aspects of the disease. A new text on the subject lists nearly 6000 references[3].

The high morbidity and mortality rates of disseminated visceral candidiasis[4] are not necessarily due to the inherent failure of antifungal chemotherapy but rather to the delay of treatment. Because of the lack of reliable non-invasive diagnostic procedures, the majority of cases are not diagnosed early enough to permit effective

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treatment. Histologic demonstration of tissue invasion or repeated positive blood cultures, which take too long, are the only definitive methods of diagnosis; negative blood cultures are not reliable for diagnosis or surveillance[5]. Although a variety of serologic tests have been described[6–8], improvements in sensitivity and practicality are clearly needed[9]; the first attempt to develop a DNA probe for *Candida albicans* was unsuccessful[10].

D-Arabinitol, a five-carbon unbranched sugar alcohol (Fig. 1), was identified by mass spectrometry (MS) as a major metabolite of several *Candida* species[11,12] and both gas chromatographic (GC) and combined GC–MS techniques were developed for the quantification of arabinitol (as the trimethylsilyl derivative) in serum and urine[11,13]. Another GC technique[14], based on per-O-acetylated aldononitrile derivatization, had some methodological problems[15]. These techniques have been applied to determine arabinitol in infected experimental animals[14,16–19] and in humans with disseminated candidiasis[11,13,16,18,20–26]. Although it was definitely established that candidiasis results in increased arabinitol concentrations in both serum and urine, the results of clinical trials were considered ambiguous by some investigators[22, 23, 25]. Controversial issues included problems due to increased serum arabinitol concentrations in the presence of renal dysfunction, and the absolute values and variability of endogenous arabinitol concentrations. An approach to the first problem was the suggestion to use arabinitol/creatinine ratios rather than just arabinitol concentrations[18,20]. The second problem was approached by developing a separation technique, using capillary GC columns, for the three pentitols[27]. Packed GC columns have been used in most published work, and thus the reported "arabinitol" concentrations were, in fact, total pentitol (arabinitol + xylitol + adonitol) concentrations (Fig. 1), leading to occasional false positives due to increased endogenous adonitol and/or xylitol concentrations in the presence of normal arabinitol[28].

An obvious improvement in diagnostic specificity would be achieved by differentiating between arabinitol concentrations of fungal and non-fungal origin. Three techniques have been described to provide enantioselectivity, each utilizing microbiological techniques prior to GC analysis and each requiring two analyses to obtain the D- and L-arabinitol concentrations[29–33]. The objective of the present work was to develop a GC–MS technique using a chiral column for the direct chromatographic separation of the enantiomers of arabinitol. In pilot studies, the technique was applied to determine D/L ratios of arabinitol in serum and urine from normal subjects, cancer patients without and with confirmed disseminated visceral candidiasis, and patients with renal dysfunction.

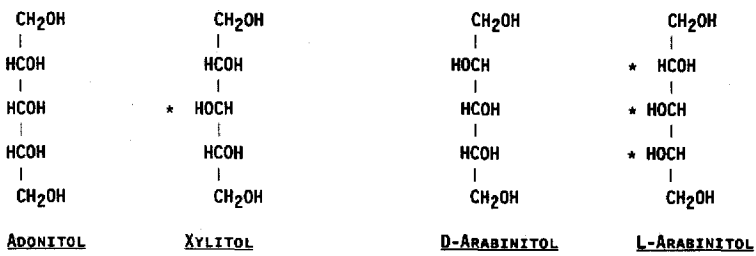

Fig. 1. Formulas of pentitols. The stars (⋆) mark the carbon atoms responsible for the differences between adonitol and xylitol and for the enantiomers of arabinitol.

EXPERIMENTAL

Chemicals

D(+)-Arabinitol, L(−)-arabinitol, adonitol and xylitol were obtained from Sigma (St. Louis, MO, U.S.A.). [$^2H_7$]D-arabinitol, 96 atom% deuterium, was purchased from MSD Isotopes (Montreal, Canada). Methanol, methylene chloride (HPLC grades), toluene (certified, ACS) and the Nalgene sterile disposable filters (0.45 μm, 30 ml) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Trifluoroacetic anhydride, in 1-ml ampules, was purchased from Supelco (Bellefonte, PA, U.S.A.).

Stock solutions of the sugar alcohols were prepared at a concentration of 1 mg/ml in methanol–water (1:1, v/v) and diluted further as needed. The concentration of the internal standard, deuterated arabinitol, was 5 μg/ml; 100 μl aliquots were added to all samples analyzed.

Sample preparation

Culturing Candida albicans in serum. Two 6-ml aliquots of a normal serum sample, in 20-ml vials, were heat deactivated at 56°C for 30 min. One sample was inoculated with *Candida albicans* (from a patient with confirmed candidiasis), the other served as a control. After incubation for 2 days the samples were filtered through 0.45-μm sterilized disposable filters. The filtrates were further prepared as serum samples.

Serum. To 200 μl serum, 100 μl of internal standard (5 μg/ml) and 500 μl hexane were added in a 75 × 10 mm culture tube. After vortexing for 10 s and centrifuging at 3000 *g* for 10 min, the aqueous layer was transferred to another culture tube and 1.8 ml acetone was added. After vortexing for 10 s and centrifuging at 3000 *g* for 10 min the supernatant was transfered to a 6-ml screw cap vial and evaporated to dryness with a stream of nitrogen in a water bath kept at 35°C.

Urine. Urine samples, usually 500-μl aliquots, were first filtered through a microconcentrator (Centricon 10; Amicon, Danvers, MA, U.S.A.) at 3000 *g* for 30 min. Aliquots (50 μl) of the filtrate were transferred to small (300-μl) vials, 100 μl of internal standard (5 ng/μl) added and the samples were then evaporated to dryness with nitrogen in a water bath kept at 35°C.

Calibration samples. Calibration samples were made by supplementing normal serum or urine with increasing quantities of D- or L-arabinitol, or both, with a fixed amount of internal standard.

Derivatization. To the dried samples, 200 μl methylene chloride and 100 μl of trifluoroacetic anhydride were added, the vials were tightly closed (Tuf Bond liners; Pierce, Rockford, IL, U.S.A.) and heated at 75°C for 20 min in a dry heating block. The samples were next allowed to cool to room temperature and the solvents were evaporated to dryness with a gentle stream of nitrogen at room temperature. The residues were dissolved in 50 μl toluene (vortex for 10 s). Aliquots of 1.0 or 1.5 μl were injected into the GC–MS system.

Gas chromatography–mass spectrometry

A combined gas chromatograph (Model 5890A, Hewlett-Packard, Avondale, PA, U.S.A.)–triple quadrupole mass spectrometer-computer (Model Trio-3, VG Masslab, Altrincham, U.K.) system was used. Glass capillary columns, 20 m or *ca.* 40

m long, 0.25 mm I.D., coated with α-perpentylated cyclodextrin were obtained from Professor W. A. König, University of Hamburg, F.R.G.[34,35]. These columns are now commercially available (Lipodex A) from Macherey-Nagel (Düren, F.R.G.) and their distributors in the U.S.A. The columns were operated isothermally at 100°C for 7 min (20 m length) or 18 min (40 m length). As discussed later, elution temperatures were increased as the columns aged. Columns were cleaned after every analysis by increasing the temperature to 150°C at 20°C/min, holding at that temperature for 4 min and then recycling to 100°C. Helium was the chromatographic carrier gas. The end of the glass capillary column was connected to the ion source via a short uncoated vitreous silica column. No helium separator was used; the GC–MS transfer line was kept at 200°C.

The mass spectrometer was operated in the positive chemical ionization mode using isobutane as the reagent gas. Tuning parameters in the ion source were adjusted to provide maximum intensity for the *m/z* 519 ion which was obtained by introducing 5 μl of derivatized D-arabinitol (0.5 μg/μl solution) through the septum inlet. Typical source conditions were: electron current 1 mA, electron energy 100 eV, multiplier 650–950 V.

Samples (1.0 or 1.5 μl size) were introduced through a split/splitless capillary inlet (splitless mode; 0.75 min valve time) kept at 200°C. Full mass spectra were obtained by scanning from *m/z* 100 to 650 in 1 s. Selected ion monitoring of *m/z* 519 and 526 were performed by scanning for 80 ms with a 20 ms delay.

D/L Ratio measurements and quantification

D/L ratios were determined from peak heights, determined by the computer or by manual measurements, or peak areas. Calibration curves were made by plotting the area ratios of D- and L-arabinitol peaks against the amounts added to the calibration samples. Separate calibration curves were obtained for D- and L-arabinitol. Extrapolation of the calibration lines to the abscissa gave the endogeneous concentrations of D- and L-arabinitol.

RESULTS

Mass spectra

Fig. 2 shows the chemical ionization (isobutane) mass spectrum of the O-trifluoroacetyl (O-TFA) derivative of D-arabinitol. The chemical ionization mass spectra of L-arabinitol, adonitol and xylitol were nearly identical to that of D-arabinitol. The base peak was at *m/z* 519, corresponding to the $(M\text{-}113)^+$ ion which results from the loss of an OCF_3CO (TFAO) group from the fully acetylated compound, $C_{15}H_7O_{10}F_{15}$. Four additional fragment peaks were found with the following identifications and relative intensities: *m/z* 613, $(M-F)^+$, <5%; *m/z* 379, $(M-2TFAO-C_2H_3)^+$, <10%; *m/z* 253, $(M-3TFAO-C_3H_4)^+$, 32%; and *m/z* 177, (M − 4TFAO − 3H), 28%. The base peak in the spectrum of the O-TFA derivative of the internal standard, fully deuterated [$^2H_7$]D-arabinitol, was at *m/z* 526.

Chromatographic separation

Fig. 3 illustrates the separation of pure D- and L-arabinitol, and also of adonitol and xylitol (1 ng each), using a relatively new column, 20 m long; the *m/z* 519 and *m/z*

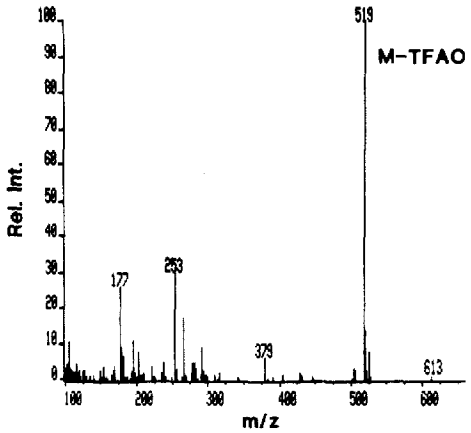

Fig. 2. Positive chemical ionization (isobutane reagent gas) mass spectrum of the O-trifluoroacetylated derivative of D-arabinitol. M = molecular ion; TFAO = OCF_3CO. Mass spectrum was obtained by scanning from *m/z* 100 to 650 in 1 s after injecting 104 ng of derivatized D-arabinitol in 1 μl of toluene.

526 (internal standard) ions were monitored. The retention times of D- and L-arabinitol were between 4 and 5 min and the separation was nearly baseline. The retention time of the internal standard was virtually identical to that of D-arabinitol; of course, the mass difference provides complete separation. Using *ca.* 40 m long columns, retention times increased to 16–18 min as illustrated later (Fig. 4). Long columns were used in the experiments with *Candida* grown in serum and for the determination of D/L ratios in urine; short columns were used in most experiments with serum. The long columns offered no advantages over the short ones, therefore, all work is now being carried out using 20 m columns.

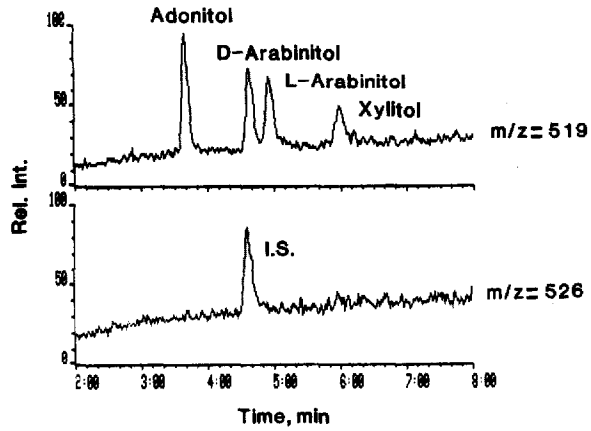

Fig. 3. Selected ion monitoring of the separation of the O-trifluoroacetyl derivatives of adonitol, xylitol and the enantiomers of arabinitol. A 1-ng amount of each was injected (in 1 μl toluene) onto a 20-m long α-perpentylated cyclodextrin glass capillary column kept isothermally at 100°C; helium was used as carrier gas. Selected ion monitoring of *m/z* 519 and 526 (corresponding to the loss of OCF_3CO group from the pentitols and internal standard, respectively) were performed by scanning for 80 ms with a 20 ms delay. I.S. refers to $[^2H_7]$D-arabinitol.

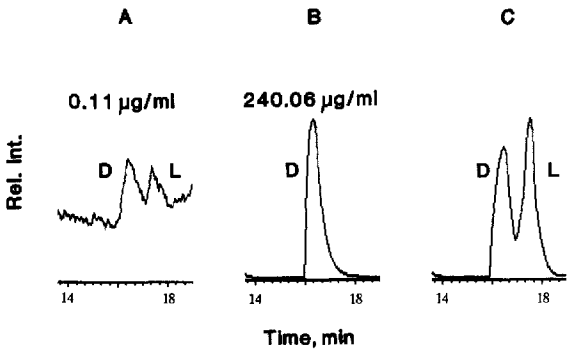

Fig. 4. Monitoring of m/z = 519 ions of: A = normal serum; B = serum in which *Candida albicans* was grown; and C = serum of B after supplementing with 340 ng L-arabinitol. Chromatographic and mass spectrometeric conditions were the same as in Fig. 3, except that a 40-m long column was used.

As the columns aged, chromatographic resolution decreased, and in many runs the separation was poorer than that shown in Fig. 3 (*e.g.*, see Fig. 6A and 6B). Separation could be improved by increasing columns temperature to 105–110°C or by temperature programming, *e.g.*, 90–100°C at a rate of 0.5°C/min and then holding for 10 min. Before substantial column deterioration 200–300 analyses could be made; we were not able to regenerate the columns.

D-Arabinitol in Candida albicans

To confirm the stereoisomeric nature of arabinitol produced by *Candida* organisms, *Candida albicans* was grown in heat-deactivated serum. The D/L ratio in the control serum was 1.8 (peak heights), the total quantity of D-arabinitol was 0.11 µg/ml (Fig. 4A). The serum filtrate, after culturing was complete, contained only D-arabinitol 240.06 µg/ml. L-Arabinitol was not detectable (Fig. 4B). The presence of D–L resolving power was proven by supplementing a 10-µl aliquot of the sample shown in Fig. 4B with a 2-µl aliquot of a 2.04 µg/µl L-arabinitol solution; the amount of injected L-arabinitol was 340 ng (Fig. 4C).

Reproducibility of serum D/L arabinitol ratio measurements

Serum samples from a healthy subject were used to compare D/L ratio determinations made by peak height and peak area measurements, and also to investigate within day and long term reproducibility. Data were obtained so that within-day replicates were made using samples prepared parallel, while analyses at other times were made using freshly prepared samples (Table I). In addition to data on within-day reproducibility of D/L ratios by peak heights (manual and computer) and peak areas, comparisons were also made between peak heights determined manually and by computer, and between peak heights and peak areas, both by computer. Long-term reproducibility data were obtained by both peak heights and areas from a set of samples analyzed four times within a 10 day period and 6 times within a 6 month period; in these comparisons, the data were calculated from averages of duplicate or triplicate measurements of each sample (Table I).

TABLE I

WITHIN-DAY AND LONG-TERM COMPARISONS OF D/L ARABINITOL RATIOS IN NORMAL SERUM BASED ON PEAK HEIGHTS AND PEAK AREAS

S.D. = Standard deviation.

Comparison		*n*	*Mean*	*S.D.*	*Range*
Peak heights					
Sample 1[a]	Computer	4	1.48	0.28	1.20–1.74
	Manual	4	1.53	0.50	1.07–2.00
Sample 2	Computer	3	1.35	0.24	1.08–1.56
	Manual	3	1.37	0.37	1.00–1.74
Peak height *vs.* areas[b]					
Sample 1[a]	Heights	7	1.50	0.28	1.20–1.92
	Areas	7	1.44	0.26	1.08–1.74
Sample 2	Heights	4	1.47	0.32	1.08–1.84
	Areas	4	1.27	0.09	1.14–1.36
Four times within 10 days[c]					
	Heights	4	1.42	0.28	1.05–1.71
	Areas	4	1.28	0.18	1.02–1.44
Six times within 6 months[c]					
	Heights	6	1.40	0.29	0.94–1.83
	Areas	6	1.66	0.33	1.15–2.12

[a] Samples 1 and 2 are duplicates prepared parallel from the same serum
[b] Both by computer
[c] All values calculated from averages of duplicate or triplicate measurements

D/L Arabinitol ratio in normal serum

The D/L profile of a normal serum is illustrated in Fig. 5 which also shows the monitoring of the internal standard; the D/L ratio was 1.6 (peak heights). The mean ± S.D. (range) serum D/L for 21 healthy subjects was 1.40 ± 0.42 (0.72–2.21) based on peak heights and 1.51 ± 0.45 (0.75–2.23) based on peak areas. For 10 cancer patients without candidiasis the values were (peak heights) 1.56 ± 0.41 (1.02–2.19). The D/L ratios of normals and candidiasis-free cancer patients were not significantly different (two-tailed *t*-test). The upper limit of normal was defined as the mean + 2S.D. Thus, serum D/L ratios > 2.24 (peak height) or > 2.42 (peak areas) were considered to be outside the normal range. D/L ratios in clinical samples were calculated from peak heights.

D/L Arabinitol ratios in clinical samples.

D/L profiles for two cases of confirmed candidiasis and two cases of renal dysfunction but no candidiasis are illustrated in Fig. 6; the internal standard profiles, normalized to 100, were omitted for clarity. In one confirmed (autopsy and blood culture) case, creatinine was high, 5.4 mg/dl (upper limit of normal is 1.5 mg/dl), and so was total arabinitol, 8.1 μg/ml. (Upper limit of normal for total arabinitol, determined by the method in ref. 13 was defined as 0.9 μg/ml.) The D/L ratio was 3.2, considerably higher than the threshold value of 2.2. The second confirmed (positive blood culture) sample contained normal creatinine, 1.3 mg/dl, and high total arabini-

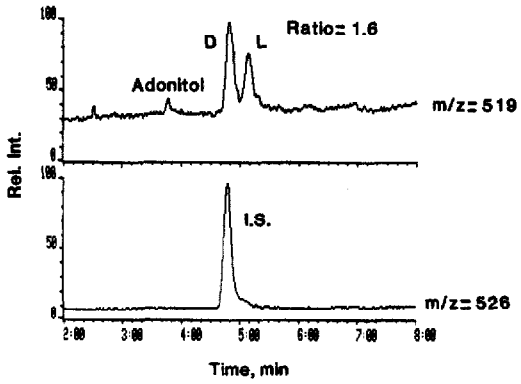

Fig. 5. Monitoring D-and L-arabinitol and the internal standard in a serum sample from a healthy subject. Starting with 200 μl serum, lipids were removed with hexane, proteins were precipitated with acetone, the dried supernatant was derivatized, the residue after evaporation was reconstituted with 50 μl toluene and 1 μl was injected. Chromatographic and mass spectrometric conditions were the same as in Fig. 3.

tol, 2.9 μg/ml; the D/L ratio was 5.3 (Fig. 6B). The D/L ratio in a serum sample from a patient on hemodialysis (no candidiasis) was 1.5 (Fig. 6C), well within the normal range; creatinine was 3.4 mg/dl, total arabinitol, 2.7 μg/ml. In a patient with renal dysfunction (creatinine 1.9 mg/dl) but without candidiasis the D/L ratio was also normal, 1.7 (Fig. 6D); total arabinitol was 0.5 μg/ml.

In a pilot study of twelve cases of confirmed (autopsy and/or blood culture) disseminated candidiasis, the D/L ratio was >2.2 (range: 2.4–14.2) in ten cases; one case was a false negative (D/L = 1.3) and another was borderline (D/L = 2.2).

D/L Ratios in urine

A D/L profile in normal urine is illustrated in Fig. 7A. Mean $\pm$ S.D. (range) values for D/L ratios in normal urine (n = 13) were 1.15 ± 0.32 (0.68–1.89) and 1.75 ± 0.4 (1.31–2.43) based on peak height and peak area measurements, respectively. Measurements were also made based on the concentrations (determined from calibration curves using peak areas) of the separated D and L enantiomers. The concentrations of D- and L-arabinitol, in μg/ml (range), were 18.45 ± 7.22 (7.6–32.1) and 12.1 ± 4.02 (4.1–20.4), the total D- and L-arabinitol concentration was 30.55 ± 10.51 (11.7–48.1). The D/L ratio was 1.55 ± 0.34 (1.11–2.14).

The upper limits of normal for the D/L ratio in urine, taken as the mean + 2S.D., were 1.79, 2.55 and 2.23 based on peak heights, peak areas, and measured quantities, respectively. D/L ratios above these values were considered outside the normal range. Fig. 7B illustrates a D/L profile in a urine sample from a patient with confirmed (autopsy and blood culture) disseminated candidiasis. The D/L ratio was 4.2; the D/L ratio in the serum of this patient was 4.0.

DISCUSSION

Several methodological problems of the earlier GC techniques have been eliminated by GC–MS techniques[27,28]: (i) the use of capillary columns to separate the

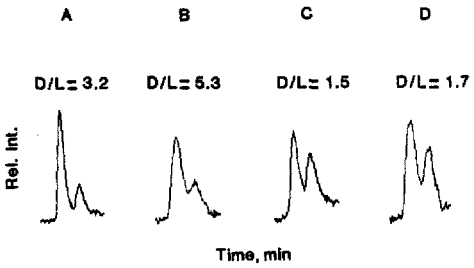

Fig. 6. Serum D- and L-arabinitol profiles in: A = confirmed disseminated candidiasis with high creatinine; B = confirmed disseminated candidiasis with normal creatinine; C = renal failure (hemodialysis); D = renal dysfunction. Sample preparation, chromatographic and mass spectrometric conditions were the same as in Figs. 3 and 5.

three pentitols, resulting in a decrease in the number of false positives due to increased endogenous adonitol and/or xylitol; (ii) the use of deuterated arabinitol as the internal standard[28] increases reliability and eliminates the need for multiple internal standards to overcome unpredictable interferences[18,20]; (iii) detection by selected ion monitoring is much more specific than by flame ionization because only ions charateristic of the analyte are monitored; and (iv) both absolute and incremental sensitivities are inherently higher in selected ion monitoring than in flame ionization detection. Yet, the arabinitol technique cannot be accepted for clinical use until the problem of differentiation of increased arabinitol due to fungal and non-fungal origin is satisfactorily resolved.

The source of endogenous arabinitol is unknown. It had been assumed[29], based on some indirect evidence[12,36,37], that mammalian metabolism yields only L-arabinitol; this, combined with the identification of the D form of arabinitol as the major metabolite of *Candida* species[11], led to the suggestion[29] that separation of the enantiomers of arabinitol may provide a means to differentiate between arabinitol of fungal and non-fungal origin.

There are at least four possible approaches to the separation of the stereoisomers of arabinitol: stereospecific derivatization, microbiological or enzymatic removal of D- or of L-arabinitol, and chromatographic separation using a chiral column.

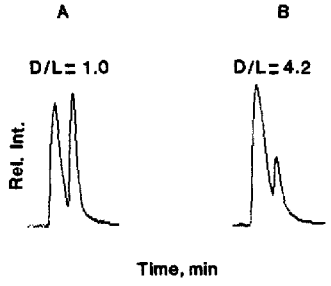

Fig. 7. Urine D- and L-arabinitol profiles in: A = normal urine; B = urine from confirmed disseminated candidiasis (D/L ratio in serum was 4.0). Starting with 500 μl urine, 50 μl of ultrafiltrate (10 000 dalton exclusion limit) was dried, derivatized, the residue after evaporation was reconstituted with 50 μl toluene and 1 μl was injected. Chromatographic and mass spectrometric conditions were the same as in Fig. 3.

Stereospecific derivatization would yield diastereoisomers which could then be separated on conventional GC columns; to our knowledge, this approach has not yet been accomplished. Using a microbiological–GC technique, D-arabinitol concentrations were determined by measuring arabinitol before and after incubation with a special strain of *Candida tropicalis* which ultilizes D-arabinitol as a sole carbon source for growth when preferred substrates are unavailable[29]. Although this technique is not practical for repetitive analyses, it was the first demonstration of the potential of using excess D-arabinitol in serum, urine, and tissues as a marker for disseminated candidiasis.

A non-chromatographic technique has been described that uses D-arabinitol dehydrogenase (ADH), isolated from *Enterobacter aerogenes*, to oxidize D-arabinitol to D-xylulose. The rate of the concomitant reduction of oxidized nicotinamide–adenine dinucleotide (NAD) was determined by following the coupled reaction between reduced nicotinamide–adenine dinucleotide (NADH) and resazurin with a spectrophotofluorimeter[30,31]. The technique lacks substrate specificity because endogenous D-mannitol is also oxidized[32]. A combined enzymatic–GC technique used ADH from *Klebsiella pneumoniae* to remove D-arabinitol from serum; arabinitol concentrations were determined in the enzyme-treated and untreated serum by GC and D-arabinitol concentrations were then calculated from the difference. D- and L-arabinitol concentrations and arabinitol/creatinine ratios were determined in serum samples from 27 normal subjects and four candidiasis patients; the latter all had significantly increased D-arabinitol[33].

Techniques based on the removal of D-arabinitol using bacterial ADH[31,33] have several disadvantages: (i) purified ADH is not commercially available; local isolation, purification and characterization is time consuming; (ii) two analyses are required for each sample, one with and one without enzyme treatment; (iii) antifungal drugs interfere with enzyme action[33], making these techniques unreliable for treatment monitoring. In addition, the enzyme-fluorimetric[31] method is not specific and provides inherently high results because only the sum of D-arabinitol and D-mannitol is determined, while the enzymatic–GC method[33] requires a 24 h incubation and lacks adequate sensitivity to determine reliably the residual L-arabinitol concentrations.

The present work describes the separation of the enantiomers of arabinitol by capillary chromatography followed by quantification using chemical ionization MS with selected ion monitoring. The work is based on the development of a new chiral stationary phase capable of separating enantiomeric carbohydrate derivatives[34,35]; glass capillary columns coated with the modified cyclodextrins are now commerically available (see Experimental). The separation of D- and L-arabinitol, and also of the other polyols, was impressive when relatively new columns were used (Fig. 3). Using 40 m long columns did not increase the resolution of these peaks; of course, retention times increased significantly (Fig. 4). The longer columns were not better than the short ones (20 m long) and their use is now discontinued. Resolution deteriorated as the columns aged (Fig. 6A and 6B), necessitating the empirical changing of column temperatures to maximize separation. Although somewhat inconvenient, this did not significantly change performance. Both column performance and useful life was significantly extended by rapidly increasing the temperature to 150°C after the elution of the arabinitol and xylitol peaks to remove additional serum constituents present and

to recondition the column. Development of fused-silica capillary columns coated with cyclodextrins will certainly make them more convenient to install and improve performance as well as life span.

Chromatographic retention times varied, sometimes by several minutes, depending on the age of the columns and the changes made in temperature programming to optimize separation. This was not a disadvantage because the nearly identical retention times of D-arabinitol and the internal standard made peak identification an easy task in the selected ion monitoring mode (Figs. 1 and 5). In contrast, variations in retention times may present serious problems in GC, particularly when small peaks must be identified within the ever present "chemical noise" patterns. Interferences from other serum constituents were observed only on a few occasions and were easy to detect.

The first test of the technique was to confirm that the metabolic product of *Candida albicans* was in fact D-arabinitol, as suggested on the basis of melting point determinations[11]. A large quantity of D-arabinitol was found in serum in which the organism was cultured; no measurable increase of L-arabinitol was detectable, confirming that *Candida albicans* produces only D-arabinitol (Fig. 4).

The technique can be applied to determine D/L ratios in serum and urine using: (i) individual concentrations of the separated enantiomers; and (ii) peak height or peak area measurements. The first method requires calibration curves, which are obtained by analyzing a set of control samples supplemented with known amounts of D- and L-arabinitol[13,27,28]. The second method is obviously much simpler as it requires no calibration curves. An important conclusion from the present work is that measuring D/L ratios alone is adequate for the confirmation of the presence of arabinitol of fungal origin; usually there is no need to quantify the individual enantiomers.

The data presented for D/L ratios in a normal serum sample (Table I) can be interpreted from several points of view. For example, the mean values based on peak heights by computer ranged from 1.40–1.50 with standard deviations of 0.28–0.29 in both the within-day and long-term reproducibility runs, including replicate analyses made on the same sample, and the comparisons of sample prepared at various times from the same original serum. Comparing peak heights obtained by computer and manually, and also peak heights against peak areas, it was concluded that D/L ratios calculated from computer-measured peak heights would be the best choice for serum.

The mean D/L ratio in healthy subjects ($n = 21$) was 1.40. This value, calculated directly from peak heights without determining concentrations, is remarkably close to the D/L ratio of 1.38 calculated from D- and L-arabinitol concentrations determined by the enzymatic–GC method[33]. These data indicate that endogeneous serum and urine arabinitol contains both the D and L forms, in contrast to the previous assumption that only L-arabinitol results from mammalian metabolism.[29]

The mean D/L ratio in serum samples from patients with various cancers but without candidiasis ($n = 10$) was 1.56 which is not significantly different from that of normals. This is in agreement with earlier results which used GC–MS and showed no significant increase in arabinitol concentrations in cancer patients without candidiasis and with normal renal function[26,27], and is in contrast to suggestions, based on GC runs, that cancer patients may have somewhat increased serum arabinitol concentrations even in the absence of candidiasis[23,33].

By taking the upper limit of normal as the mean + 2S.D., serum D/L ratios > 2.2

were considered indicative of disseminated candidiasis. Of the twelve confirmed cases analyzed, ten were positive by this criterion, one was a false negative and one was borderline. The four confirmed cases studied by the enzymatic–GC method[33] would all be positive on the basis of this criterion using the given concentrations (μg/ml) to calculate the D/L ratios.

The D/L arabinitol ratio remained normal in the absence of candidiasis even in the presence of considerably increased arabinitol concentrations due to non-fungal origin (Fig. 6C, 6D). The D/L arabinitol ratio increased in the presence of disseminated candidiasis even when creatinine was normal (Fig. 6B). Not enough analyses were made in these groups to permit statistical evaluation.

Sample preparation for urine is much easier than for serum because of the very low protein content. The specificity of the selected ion monitoring technique permitted the injection of TFA-derivatized evaporated ultrafiltrates directly without encountering interferences. Because of the relatively large amounts of arabinitol present in urine, the peaks were larger and more symmetrical than in serum. It was concluded from comparative reproducibility measurements that the best data for D/L ratios would be provided by peak area measurements. The mean D/L ratio in normal urine (n = 13) was 1.75 using peak area measurements without determining concentrations and 1.55 using the concentrations of the individual enantiomers (determined using calibration curves). The mean D/L arabinitol ratios of serum and urine were quite close: 1.40 and 1.15 using peak heights and 1.51 and 1.75 using peak areas, respectively. These agreements are remarkable, particularly when one considers that the concentration of arabinitol is of the order of 60 times larger in normal urine than in normal serum. The D/L ration in the serum and urine of a patient with disseminated visceral candidiasis was also nearly identical (Fig. 7B).

The combination of chiral separation with selected ion monitoring of the base peaks of the pentitols and that of the deuterated internal standard obtained in chemical ionization offers several advantages: (i) only one sample needs to be prepared and analyzed; (ii) in addition to the enantiomers of arabinitol, adonitol and xylitol can also be analyzed at the same time; (iii) peak identification, based on relative retention times with respect to the internal standard, is simple; (iv) deuterated arabinitol is an almost ideal internal standard (but it cannot be used by GC methods); (v) because only two masses are monitored, most interferences are eliminated; (vi) there is adequate sensitivity to quantify the individual stereoisomers; (vii) the technique is relatively fast: 18 samples/day can be analyzed conveniently; (viii) the method is applicable to both serum and urine.

The following conclusions are made: (i) the separation of the enantiomers of arabinitol in serum and urine by using chiral columns has been accomplished; (ii) it was confirmed that the arabinitol produced by *Candida albicans* grown in serum is of the D configuration; (iii) the determination of D/L ratios of endogenous arabinitol in serum and urine has shown that they are virtually the same despite the large differences in absolute concentrations; (iv) from a pilot study with confirmed cases of disseminated visceral candidiasis, changes in D/L ratios can be used to differentiate between increased arabinitol due to *Candida* organisms and that due to non-fungal origin; (v) endogenous serum and urine concentrations of both D- and L-arabinitol is a rather fixed ratio over a broad range among normal subjects, cancer patients and those with renal dysfunction casting a considerable doubt on the concept that human

tissues make only L-arabinitol; and (vi) measuring the D/L arabinitol ratios from peak heights or areas, without determining the concentrations of the separated enantiomers, is adequate for the differentiation.

Serum and urine samples from larger number of population of normal subjects and cancer and other patients without candidiasis should be analyzed to determine more accurately the upper limit of normal. An important question that remains to be studied is whether D/L arabinitol ratios in serum or urine provide a more sensitive, specific, and timely indication of the presence or onset of disseminated visceral candidiasis.

The technique described should increase the sensitivity and specificity of the "Arabinitol" method for the diagnosis of disseminated visceral candidiasis, for the monitoring of antifungal chemotherapy and, perhaps most importantly, for the longitudinal monitoring of patients at risk of developing the disease. Earlier diagnosis could lead to more efficient drug treatment with reduced toxicity. Other potential areas of application included the more detailed characterization of the polyol production of *Candida* and other yeast species and the study of the origin of the human endogenous arabinitol enantiomers.

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REFERENCES

1 R. Rubin and L. Young (Editors), *Critical Approach to Infection in the Compromised Host*, Plenum, New York, 2nd ed., 1988.
2 R. Horn, B. Wong, T. Kiehn and D. Armstrong, *Rev. Infect. Dis.*, 7 (1985) 646.
3 F. Odds, *Candida and Candidosis*, Bailliere, London, 2nd ed., 1988.
4 T. Eilard, *Scand. J. Infect. Dis.*, 19 (1987) 145.
5 G. Bodey, *Am. J. Med.*, 77(4D) (1984) 13.
6 D. Poulain, J. Fruit, L. Fournier, E. Deiocas and A. Vernes, *Eur. J. Clin. Microbiol.*, 5 (1986) 427.
7 M. Ness, Wm. Vaughan and G. Woods, *J. Infect. Dis.*, 159 (1989) 495.
8 R. Matthews and J. Burnie, *J. Clin. Microbiol.*, 26 (1988) 459.
9 J. Bennet, *Rev. Infect. Dis.*, 9 (1987) 398.
10 L. Cheung and J. Hudson, *Diagn. Microbiol. Infect. Dis.*, 10 (1988) 171.
11 T. Kiehn, E. Bernard, J. Gold and D. Armstrong, *Science (Washington, D.C.)*, 206 (1979) 577.
12 E. Bernard, K. Christiansen, S. Tsang, T. Kiehn and D. Armstrong, *J. Clin. Microbiol.*, 14 (1981) 189.
13 J. Roboz, R. Suzuki and J. F. Holland, *J. Clin. Microbiol.*, 12 (1980) 594.
14 L. deRepentigny, R. Kuykendall and E. Reiss, *J. Clin. Microbiol.*, 17 (1983) 1166.
15 B. Wong, J. Roboz, E. Bernard, R. Suzuki, J. F. Holland and D. Armstrong, *J. Clin. Microbiol.*, 21 (1985) 478.
16 R. Eng, H. Cheml and M. Buse, *J. Infect. Dis.*, 143 (1981) 677.
17 B. Wong, E. Bernard, G. Gold, D. Fong, A. Silber and D. Armstrong, *J. Infect. Dis.*, 146 (1982) 346.
18 B. Wong, E. Bernard, J. Gold, D. Fong and D. Armstrong, *J. Infect. Dis.*, 146 (1982) 353.
19 L. deRepentigny, R. Kuykendall, F. Chandler, J. Broderson and E. Reiss, *J. Clin. Microbiol.*, 19 (1984) 804.
20 J. Gold, B. Wong, E. Bernard, T. Kiehn and D. Armstrong, *J. Infect. Dis.*, 147 (1983) 504.
21 J. Roboz, *Recent Dev. Mass Spectrom. Biochem. Med.*, 7 (1983) 1.

22 C. Wells, M. Sirany and D. Blazevic, *J. Clin. Microbiol.*, 18 (1983) 353.
23 L. deRepentigny, L. Marr, J. Keller, A. Carter, R. Kuykendall, L. Kaufman and E. Reiss, *J. Clin. Microbiol.*, 21 (1985) 972.
24 A. Deacon, *J. Clin. Pathol.*, 39 (1986) 842.
25 E. Holak, J. Wu and S. Spruance, *Mycopathologia*, 93 (1986) 99.
26 J. Roboz, D. Kappatos and J. F. Holland, *Cancer*, 28 (1987) 224.
27 J. Roboz, D. Kappatos, J. Greaves and J. F. Holland, *Clin. Chem. (Winston-Salem, N.C.)*, 30 (1984) 1611.
28 J. Roboz, D. Kappatos and J. F. Holland, *Eur. J. Clin. Microbiol.*, 6 (1987) 708.
29 E. Bernard, B. Wong and D. Armstrong, *J. Infect. Dis.*, 151 (1985) 711.
30 K. Soyama and E. Ono, *Clin. Chim. Acta*, 149 (1985) 149.
31 K. Soyama and E. Ono, *Clin. Chim. Acta*, 168 (1987) 259.
32 K. Soyama and E. Ono, *Clin. Chem. (Winston-Salem, N.C.)*, 34 (1988) 432.
33 B. Wong and K. Brauer, *J. Clin. Microbiol.*, 26 (1988) 1670.
34 W. Konig, S. Lutz and G. Wenz, *Angew. Chem. Int. Ed. Engl.*, 27 (1988) 979.
35 W. Konig, P. Mischnick-Lubecke, B. Brassat, S. Lutz and G. Wenz, *Carbohydr. Res.*, 183 (1988) 11.
36 O. Touster and S. Harwell, *J. Biol. Chem.*, 230 (1958) 1031.
37 O. Touster and D. Shaw, *Physiol. Rev.*, 42 (1962) 181.