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ENANTIOSELECTIVE MEASUREMENT OF THE *CANDIDA* METABOLITE D-ARABINITOL IN HUMAN SERUM USING MULTI-DIMENSIONAL GAS CHROMATOGRAPHY AND A NEW CHIRAL PHASE

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SUMMARY

A multi-dimensional gas chromatographic method was developed to measure the *Candida* metabolite D-arabinitol enantioselectively in human serum The heptafluorobutyrate derivatives of D-arabinitol, L-arabinitol and ribitol (internal standard) were separated from other serum constituents with a 60 m × 0 32 mm fused-silica SPB-5 precolumn, and (after intermediate cold trapping) they were separated from each other with a 25 m × 0 25 mm fused-silica column coated with a new bonded chiral phase Replicate analyses of spiked human sera showed that D-arabinitol could be quantified accurately and precisely The D- and L-arabinitol concentrations in 24 normal adult sera were 0 20 ± 0 053 and 0 11 ± 0 040 μ/ml, respectively, and the D- and L-arabinitol/creatinine ratios were 0 023 ± 0 011 and 0 012 ± 0 0051, respectively (mean ± S D) In a patient with *Candida albicans* fungemia, the D-arabinitol/creatinine ratios rose early during infection and fell with successful treatment, whereas L-arabinitol/creatinine ratios did not change significantly This enantioselective analytical method is more practical than earlier ones, it should facilitate further investigation of D-arabinitol as a diagnostic marker for candidiasis

INTRODUCTION

Candida albicans, C tropicalis and *C parapsilosis* (the species responsible for almost all human infections) produce large amounts of the pentitol D-arabinitol in vitro [1–3], and there is considerable evidence that *Candida* also produces large amounts of D-arabinitol in vivo Earlier studies have established that arabinitol appearance in the body is proportional to the arabinitol/cre-

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atinine concentration ratios in serum or urine [4] and that the arabinitol/creatinine ratios rose directly in proportion to tissue colony counts in experimentally infected rats [5]. Moreover, most patients with invasive candidiasis have higher serum arabinitol concentrations [1,2,6] and higher serum arabinitol/creatinine ratios [6] than uninfected controls These observations indicate that arabinitol is a quantitative diagnostic marker for invasive candidiasis, a disease that often cannot be recognized antemortem by traditional diagnostic methods

One limitation of these earlier studies is that D- and L-arabinitol were not differentiated This is important because *Candida* produces only D-arabinitol [1,3], whereas known mammalian metabolic pathways produce only L-arabinitol [7] We therefore developed combined microbiologic–gas chromatographic (GC) [8] and enzyme–GC [9] methods to measure D-arabinitol enantioselectively, and we found that almost all of the excess arabinitol in the tissues, urine and serum of animals and humans with invasive candidiasis was D-arabinitol These studies strongly suggested that the diagnostic sensitivity and accuracy of body fluid arabinitol measurements can be improved by using enantioselective analytical methods, but the enantioselective methods were quite cumbersome

In an effort to develop a more practical analytical method, we examined the abilities of chiral GC columns to separate D- from L-arabinitol Two commercialslly available chiral columns were unsatisfactory, but excellent results were obtained when the fluoroacyl derivatives of D- and L-arabinitol were analyzed with columns coated with a new bonded chiral stationary phase This report describes a multi-dimensional method in which conventional and chiral GC columns were used together to quantify D-arabinitol in serum, and it reports the serum D- and L-arabinitol levels in 24 healthy adults and a patient with *C albicans* fungemia

EXPERIMENTAL

Chemicals and reagents

D-Arabinitol, L-arabinitol, ribitol, xylitol, D-mannitol, glucitol and galactitol were obtained from Sigma (St Louis, MO, U S A), derivatizing reagents were obtained from Sigma or Pierce (Rockford, IL, U S A) and HPLC-grade solvents were obtained from Fisher Scientific (Pittsburgh, PA, U S A)

Instrumentation

A Model Sigma 2000 gas chromatograph (Perkin Elmer, Norwalk, CT, U S A) equipped with a split–splitless capillary injector, two flame ionization detectors and a microcomputer-based data system (PC integrator, Nelson Analytical, Cupertino, CA, U.S A) was used The instrument was modified to perform multi-dimensional analyses by installing two column switching valves

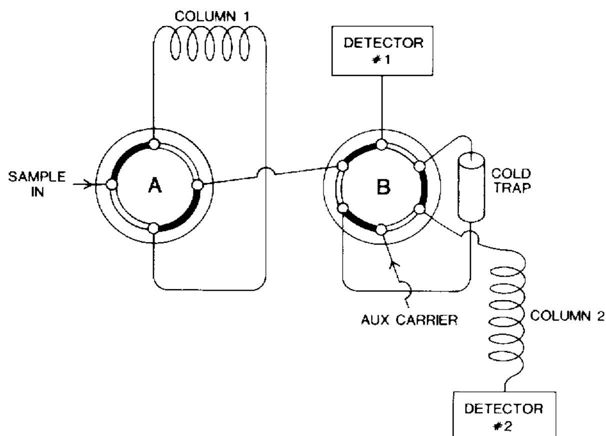

Fig 1 Flow path diagram for multi-dimensional GC Column switching valves were used for heart-cutting (B) and for backflushing column 1 (A), and a liquid CO_2 cold trap was used to refocus selected components into a narrow band before they were introduced into column 2 Flow paths within the valves shown in black were open originally and closed after switching, and those shown in white were closed originally and open after switching Column 1 was a 60 m×0 32 mm fused-silica column coated with 1 0 μm SPB-5 and column 2 was a 30 m×0 25 mm fused-silica column coated with 0 25 μm Chiral-NEB

with 0 25 mm I D flow paths (Valco Instruments, Houston, TX, U S A) and a liquid CO_2 cold trap (Scientific Glass Engineering, Houston, TX, U S A) in the column oven (see Fig 1) A 25 cm×0 25 mm I D fused-silica tubing coated with 0 25-μm bonded methylsilicone was used as the trap, and 0 22-mm uncoated deactivated fused-silica tubing was used for transfer lines

Initial studies

We first examined the abilities of fused-silica GC columns coated with three chiral stationary phases to separate D-arabinitol, L-arabinitol, xylitol and ribitol as their trimethylsilyl (TMS) ether, peracetate, trifluoroacetate (TFA), pentafluoropropionate (PFP) and/or heptafluorobutyrate (HFB) derivatives The TMS and peracetate derivatives were prepared as previously described [10], the TFA derivatives were prepared with N-methyl-bis(trifluoroacetamide) in pyridine and the PFP and HFB derivatives were prepared with PFP-anhydride and HFB-anhydride, respectively, in pyridine or acetonitrile [11]

The chiral GC columns examined were (i) a 25 m×0 25 mm fused-silica column coated with 0 12-μm L-valine-*tert* -butylamide coupled to polysiloxane (Chirasil-L-Val, Chrompack, Raritan, NJ, U S A), (ii) a 50 m×0 22 mm fused-silica column coated with 0 12-μm XE-60-S-valine-*S*-α-phenylethylamide

(Chrompack), and (iii) a 25 m×0 32 mm fused-silica column coated with 0 25 μm bonded polysiloxanes with (*S*)-N-1-(napthyl)ethyl-4-allyloxybenazamide side-chains [12] (Chiral-NEB, obtained from C A Rouse and M L Lee, Chemistry Department, Brigham Young University, Provo, UT, U S A)

Multi-dimensional GC method

The best separations were obtained when the HFB derivatives were analyzed with the Chiral-NEB column, but the column was severely overloaded when we attempted to analyze derivatized whole serum without inlet splitting

We therefore used a 60 m×0 32 mm fused-silica capillary column coated with 1 0-μm bonded 5% phenylmethylsilicone (SPB-5, Supelco, Bellefonte, PA, U S A) to separate D-arabinitol, L-arabinitol and ribitol from other serum components prior to separating these pentitols from each other with a 30 m×0 25 mm fused-silica capillary coated with 0 25 μm Chiral-NEB The carrier gas was helium at linear flow-rates of 40 cm/s in the SPB-5 precolumn and 24 cm/s in the Chiral-NEB column Samples of 2 μl were injected with the sample inlet at 225°C and the septum purge (5 ml/min) and inlet splitter (40 ml/min) valves closed for the first 30 s after injection The column oven temperature was 120°C for the first min, after which it was raised to 135°C at 3°C/min Compounds eluting from both columns were detected by flame ionization (250°C)

When the HFB derivatives of a standard polyol mixture were analyzed with the precolumn alone, the retention times were 7 31 min for erythritol, 8 10 min for xylitol, 8 58 min for DL-arabinitol and ribitol (which eluted together), 9 22 min for galactitol, 9 83 min for glucitol and 10 38 min for D-mannitol Based on these results, the cold trap was cooled at 7 4 min, components eluting from the precolumn between 8 40 and 8 95 min were transferred to the trap, and the Chiral-NEB column analysis was begun by stopping the flow of CO_2 to the trap at 9 0 min The precolumn was backflushed after 11 min to remove late-eluting compounds that might interfere with subsequent analyses Total analysis time was 20 min, after which the oven was heated to 225°C for 5 min to purge additional retained compounds

Preparation of serum samples

Serum samples were prepared for analysis as follows Ribitol (0 2 ml of 10 μg/ml aqueous solution) was added as internal standard to 0 20 ml of serum, proteins were precipitated with 1 0 ml acetone, and the supernatants (1000 *g* for 8 min) were transferred to 3-ml glass vials and evaporated to dryness in a stream of nitrogen at 65°C The HFB derivatives were formed by adding 0 05 ml HFB-anhydride and 0 20 ml acetonitrile, sealing with PTFE and heating to 50°C for 20 min

Quantitation

D-Arabinitol and L-arabinitol were quantified from their relative peak areas compared to ribitol Whenever serum samples were analyzed, the retention times of the compounds of interest were determined from a standard mixture, and the relative response factors were determined from a normal human serum to which 10 μg/ml D- and L-arabinitol had previously been added

Accuracy and precision

We performed ten replicate analyses on different days of normal human serum to which 0, 0 2, 1 0, 4 0 and 10 0 μg D-arabinitol per ml had previously been added to assess the accuracy and precision of the D- and L-arabinitol measurements

Serum samples studied

D-Arabinitol and L-arabinitol were measured in the serum of 24 healthy adults, and the results were compared to those obtained earlier by the combined enzyme–GC method [8] D-Arabinitol and L-arabinitol were also measured in eleven sera from a patient with leukemia who developed *C albicans* fungemia during an episode of neutropenia induced by cytotoxic chemotherapy These sera were obtained before the infection developed, during the early fungemic stage and during and after successful treatment with amphotericin B

Creatinine was measured with an autoanalyzer, and the D- and L-arabinitol/creatinine ratios were calculated as previously described [4] to correct for the effects of renal function

RESULTS

Initial studies

We were unable to separate D- and L-arabinitol as their TMS, peracetate or TFA derivatives with the Chirasil-L-Val column, as their TMS or peracetate derivatives with the Chiral-NEB column or as their TMS derivatives with the XE-60-S-valine-*S*-α-phenylethylamide column The XE-60-S-valine-*S*-α-phenylethylamide column resolved the peracetate derivatives of D- and L-arabinitol, but the retention times were very long ($>$30 min) and the peaks were very broad when the analyses were performed at the maximum temperature tolerated by this phase (175°C, helium at 22 cm/s, split injection) This column also separated the TFA and HFB derivatives of D- and L-arabinitol, but the peaks tailed to a considerable degree and the column was easily overloaded

The TFA, PFP and HFB derivatives D- and L-arabinitol were clearly separated by the Chiral-NEB column For example, the retention times of the HFB derivatives of ribitol, D-arabinitol, L-arabinitol and xylitol were 12 97, 13 67, 13 76, and 14 14 min, respectively (helium at 20 cm/s, splitless injection, oven

temperature 80° C for 2 min, then increased to 100° C at 20° C/min and then to 150° C at 4° C/min) Equivalent separations were obtained at lower temperatures when the TFA and PFP derivatives were analyzed with the same column We used the HFB derivatives in subsequent studies because they were more stable than the other fluoroacyl derivatives

Multi-dimensional GC analyses

In a typical multi-dimensional analysis, ribitol, D-arabinitol and L-arabinitol eluted from the Chiral-NEB column as well separated and symmetrical peaks at 17 50, 18 57 and 18 75 min, respectively, after the start of the precolumn separation (see Fig. 2)

In ten replicate analyses of normal human serum to which known amounts of D-arabinitol were added, the calibration curves were linear (mean $r = 0$ 998), the mean observed D-arabinitol concentrations differed from the expected amounts [mean y-intercept of the linear regression lines (0 16 μg/ml) + amount added] by no more than 0 04 μg/ml or 2%, and the standard deviations were no more than 0 05 μg/ml or 12%

D-Arabinitol and L-arabinitol were measurable in all serum samples studied, the lowest concentrations were 0.11 μg D-arabinitol and 0 05 μg L-arabinitol per ml In the 24 normal adult sera, the mean ± S D D-arabinitol concentration was 0 20 ± 0 053 μg/ml and the mean ± S D L-arabinitol concentration

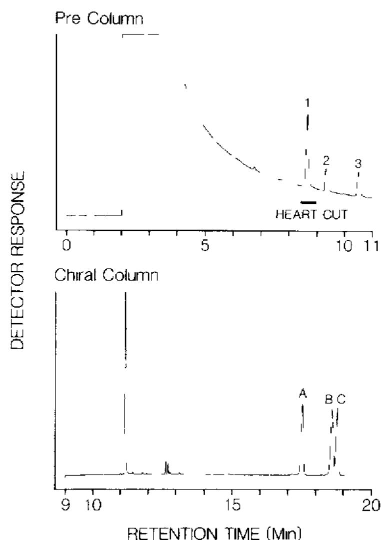

Fig 2 (Top panel) Precolumn chromatogram (from sample injection through backflushing) of the HFB derivatives of a mixture of equal amounts of several polyols (Bottom panel) Chiral-NEB column chromatogram (from turning off the cold trap to end of the analysis) of components that eluted from the precolumn between 8 40 and 8 95 min (heartcut) Peaks 1 = D-arabinitol, L-arabinitol and ribitol, 2 = galactitol, 3 = D-mannitol, A = ribitol, B = D-arabinitol, C = L-arabinitol

TABLE I

SERUM D- AND L-ARABINITOL LEVELS IN NORMAL ADULTS AND A PATIENT WITH *CANDIDA* FUNGEMIA AS DETERMINED BY MULTI-DIMENSIONAL CHIRAL GAS CHROMATOGRAPHY

Sample	D-Arabinitol (μg/ml)	L-Arabinitol (μg/ml)	D-Arabinitol/creatinine ratio	L-Arabinitol/creatinine ratio
Normal adults[a]	0 20±0 053	0 11±0 040	0 023±0 011	0 012±0 0051
Infected patient[b]				
16 May	0 22	0 15	0 037	0 025
22 May	0 09	0 06	0 018	0 012
1 June	0 16	0 06	0 027	0 009
3 June	0 71	0 10	0 10	0 014
9 June	10 8	0 45	0 83	0 035
12 June	2 2	0 30	0 24	0 033
14 June	0 92	0 23	0 12	0 029
16 June	0 61	0 21	0 068	0 023
18 June	0 52	0 18	0 052	0 018
19 June	0 59	0 22	0 054	0 020
29 June	0 61	0 24	0 061	0 024

[a]Mean ±S D ($n=24$)
[b]*C albicans* fungemia occurred on 2 June, amphotericin B therapy was begun on 5 June and the patient was discharged home on 19 June

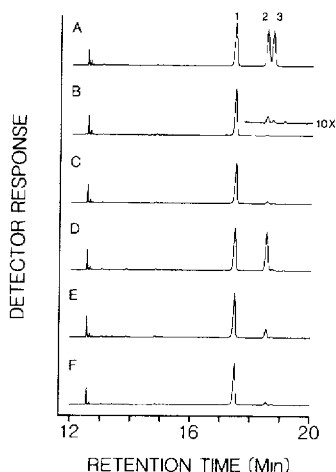

Fig 3 Chiral-NEB column chromatograms (12–20 min only) of (A) normal human serum to which 10 μg/ml D- and L-arabinitol were added and (B–F) sera obtained from the patient with *C albicans* fungemia on June 2 (B), June 4 (C), June 9 (D), June 12 (E) and June 14 (F) Peaks 1 = ribitol, 2 = D-arabinitol, 3 = L-arabinitol

was 0 11 ± 0 040 μg/ml The mean ± S D D- and L-arabinitol/creatinine ratios were 0 023 ± 0 011 and 0 012 ± 0 0051, respectively When these samples were analyzed by the enzyme–GC method [9], the mean ± S D D- and L-arabinitol concentrations were 0 21 ± 0 053 (p = 0 095 versus current results by Student's paired t-test) and 0 15 ± 0 058 (p < 0 0001), respectively

The D- and L-arabinitol results in the normal adults and the patients with *C albicans* fungemia are shown in Table I, and illustrative chromatograms are shown in Fig 3 This patient's D-arabinitol/creatinine ratios were normal before the infection developed, they were markedly elevated shortly after fungemia was recognized and treatment was begun and they declined as she improved with further therapy In contrast, there was little change in the L-arabinitol/creatinine ratios during the interval studied

DISCUSSION

We developed the multi-dimensional GC method described in this report because there were practical problems with the methods previously available for measuring D-arabinitol enantioselectively The combined microbiologic–GC method requires a 24-h incubation step, antifungal drugs can interfere with some analyses and the GC method was insufficiently sensitive to quantify D-arabinitol in most serum specimens [8] Because of these problems, we developed a combined enzyme–GC method that is sensitive enough to quantify D-arabinitol in serum, that is unaffected by antifungal drugs and that can provide results within a few hours However, this technique requires that specimens be depleted of D-arabinitol with a bacterial enzyme that must be extracted from cultures of *Klebsiella pneumonia* and then extensively purified As well, each specimen must be analyzed twice by GC to determine the D-arabinitol concentration [9]

A more straightforward approach is to separate D- and L-arabinitol chromatographically using a chiral stationary phase, but satisfactory chiral columns were unavailable until recently Chirasil-L-Val failed to resolve D-and L-arabinitol as any of the derivatives we tried XE-60-S-valine-*S*-α-phenylethylamide separated D- and L-arabinitol as their peracetate, TFA and HFB derivatives This confirmed the results of Konig and Benecke [13], who resolved the TFA derivatives of D- and L-arabinitol with a 15-m glass column coated with XE-60-S-valine-*R*-α-phenylethylamide However, the column we used separated D- and L-arabinitol only minimally, and there was considerable peak tailing Another problem is that late-eluting sample components cannot be removed from XE-60-S-valine-*S*-α-phenylethylamide columns by heating above 175°C or by rinsing with solvents We therefore concluded that XE-60-S-valine-*S*-α-phenylethylamide columns could not be used to measure D-arabinitol in complex biologic samples Konig et al [14] also recently described

excellent separations of the TFA derivatives of D- and L-arabinitol using glass capillary GC columns coated with pentylated cyclomaltohexose

Chiral-NEB is one of several chiral phases whose syntheses were recently described by Bradshaw et al [12] We found that fused-silica columns coated with Chiral-NEB separated the fluoroacyl derivatives of D- and L-arabinitol better than the other chiral columns we studied Chiral-NEB columns are also practical to use because they can be operated at temperatures as high as 280°C and they can be rinsed with solvents Late-eluting sample components can therefore be removed by purging at high temperatures or by solvent rinsing, these columns should therefore last as long as fused-silica columns coated with conventional bonded phases

The only problem we encountered was that Chiral-NEB columns have insufficient sample capacity to permit analysis of whole serum without inlet splitting It was therefore necessary to use a thick-film fused-silica precolumn to isolate the compounds of interest before they were further separated with the Chiral-NEB column We used a 60 m×0 32 mm SPB-5 column for this purpose because we use this column in other analyses, but other thick-film precolumns can also be used For example, we obtained equivalent results with a 25 m×0 32 mm fused-silica column coated with 1 2-μm of bonded methylsilicone (CP-Sil 5CB, Chrompack)

When a high-capacity thick-film precolumn and a Chiral-NEB column were used together in a multi-dimensional GC analysis, D-arabinitol was clearly separated from all other constituents of human serum (including L-arabinitol) This multi-dimensional method was sensitive enough to quantify D- and L-arabinitol in all serum samples examined, and the results were accurate and reproducible. The D-arabinitol concentrations in 24 normal sera determined by the multi-dimensional method did not differ from those obtained by the enzyme–GC method, but the L-arabinitol concentrations were slightly lower (mean difference 0 039 μg/ml) This was probably due to incomplete separation of DL-arabinitol from another trace component in the enzyme-treated sera

Lastly, multi-dimensional GC analyses showed that the serum D-arabinitol levels rose substantially in a patient with *C albicans* fungemia, whereas the L-arabinitol levels changed very little This finding confirms the results obtained earlier with the microbiologic–GC and enzyme–GC methods, and it supports our view that use of enantioselective analytical methods should result in improved ability to diagnose invasive candidiasis Availability of the more practical enantioselective analytical method described in this report should facilitate further investigation of D-arabinitol as a diagnostic marker for candidiasis

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