Journal of Chromatography, 525 (1990) 169–175
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5039

Note

Determination of monosaccharides by high-performance liquid chromatography in systemic candidosis

A. ZATON*

Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad del País Vasco, Aptdo 450, Vitoria-Gasteiz (Spain)

and

G. QUINDOS and J. PONTON

Departamento de Microbiología e Inmunología, Facultad de Medicina y Odontología, Universidad del País Vasco, Bilbao (Spain)

(First received March 3rd, 1989; revised manuscript received September 19th, 1989)

Disseminated candidosis remains a diagnostic problem because there is no pathognomonic symptomatology, and conventional microbiological methods, such as blood cultures, are usually negative. Moreover, methods for detection of antibody and antigen, although numerous, lack specificity and sensitivity [1, 2]. Explanations for the low diagnostic value of such procedures may be the presence of anti-*Candida albicans* antibodies or antigenaemia in healthy subjects induced by mucocutaneous colonization (mainly gastrointestinal and genitourinary tracts) by *C. albicans* and by the use of complex mixtures of antigens usually from the yeast phase, in serodiagnostical methods [3, 4].

Gas chromatographic (GC) studies have suggested that patients with invasive candidosis may be distinguished by the presence of elevated serum concentrations of D-arabinitol [5, 6] and/or mannose [7, 8]. This approach was originated by Miller et al. [9], who identified unusual peaks in the sera from patients with systemic candidosis.

Because GC is time-consuming, and because high-performance liquid chromatography (HPLC) appears to be more rapid and convenient for the separation of mono- and oligosaccharide isomers [10, 11], the aim of this study was

to develop a method for the quantification of mannose and other candidal metabolites or components (monosaccharides) in the sera of patients or experimental animals during the course of systemic *Candida* infection.

EXPERIMENTAL

Materials

Acetonitrile and ethanol for HPLC were obtained from Scharlau (Barcelona, Spain). Water was double-distilled, deionized and passed through Norganic cartridges obtained from Millipore (Bedford, MA, U.S.A.). Reference aldoses, ketoses and alditols were purchased from Sigma (St. Louis, MO, U.S.A.). *Candida albicans* E-139 (serotype A) was originally isolated from a patient. New Zealand white rabbits were obtained from Biocentre (Barcelona, Spain).

Standards

Standard solutions of glycerol, L-rhamnose, D-ribose, *meso*-erythritol, D-xylose, D-ribitol, D-xylitol, L-arabinose, L-arabinitol, N-acetyl-D-glucosamine, D-fructose, D-mannose, D-glucose, D-mannitol, D-galactitose, D-galactitol, myo-inositol, D-maltose, D-lactose and D-trehalose (see below) were prepared at a concentration of 400 μg/ml of eluent. These materials were chromatographed individually to obtain appropriate response factors and relative retention times. A mixture of *meso*-erythritol, D-xylose, D-ribitol, L-arabinitol, N-acetyl-D-glucosamine, D-fructose, D-mannose, L-glucose and D-galactitol was then prepared and chromatographed at the beginning of each experiment (see Fig. 1A). The concentrations of carbohydrates in unknown samples were determined by comparison with standards.

Experimental model

Rabbits were injected intravenously with *C. albicans* germ tubes or yeasts ($1 \cdot 10^8$ or $5 \cdot 10^8$ cells). The different morphological phases were obtained in Lee's synthetic medium as previously described [12]. Blood samples were obtained daily until the death of the animal. Sera were stored at $-70\,^{\circ}C$ until tested.

For solubilization of the sample in the mobile phase and serum–protein precipitation, 0.5 ml of acetonitrile was added to 0.5 ml of each serum and mixed for 2 min. After standing for 10 min each sample was filtered through a Millipore HV filter (0.45 μm). A 0.5-ml aliquot of the filtrate was evaporated to dryness. The protein-free sera were dissolved in 25 μl of water, and 10-μl aliquots of these solutions were injected into the liquid chromatograph.

Human subjects

Sera from patients with systemic candidosis and from control subjects were collected at the Hospital Civil de Bilbao and kept at $-70°C$ until tested. Clinical data have been described elsewhere. Ethanol (1 ml) was added to each serum sample (1 ml). The mixtures were mixed vigorously for 2 min.

Chromatography

The apparatus consisted of a 515 pump, a U6K universal injector and a Model R-401 differential refractometer. Peak-area integration was made with a 740 data module, all from Waters Assoc. (Milford, MA, U.S.A.). The system was equipped with a Waters Assoc. μBondapak carbohydrate analysis column (300 mm × 3.9 mm I.D.) protected by a CN Guard-Pak cartridge. All these materials were obtained from Waters Assoc. The column was equilibrated at room temperature with acetonitrile–water. In all experiments the flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

This method allowed the separation of several sugars and polyols. The retention times are shown in Table I. The best resolution of the first ten compounds was obtained with solvent I (acetonitrile–water, 83.7:16.3, v/v) but a better resolution of the later compounds was obtained with solvent II (acetonitrile–water, 80:20, v/v). The order of elution is independent of the solvent used. Chromatograms of the standards, in solvents I and II, are shown in Fig. 1a and b, respectively. These standards permitted the identification and quantification of carbohydrates in serum. Dose–response curves of glycerol and mannose standard solutions showed that 0.001 μl of glycerol and 20 μg of mannose could be determined in the biological samples, under these conditions.

Determinations of monosaccharides were made in sera from rabbits before and after inoculation with the two morphological phases of *C. albicans*, in sera from patients with candidosis and in control subjects. In the experimental model, survival time ranged between three days (animals infected with 10^8 cells) and less than two days (animals infected with $5 \cdot 10^8$ cells). *C. albicans* was recovered from all organs studied post-mortem, i.e. spleen, lung, kidney, liver and blood.

Typical elution patterns of monosaccharides in rabbit serum before and after infection are shown in Fig. 2. A major interfering peak was observed in the arabinose, arabinitol, N-acetylglucosamine and fructose elution region, 15–18 min after the beginning of elution. No treatment of the sera was found to eliminate this peak. However, the method appeared satisfactory for analysing glycerol, erythritol, ribitol, mannose, glucose and galactitol.

Sera from infected rabbits showed a significant increase in glycerol and mannose concentrations (Fig. 2, Table II). This increase was higher in sera from

TABLE I

RETENTION TIMES OF MONOSACCHARIDES AND OLIGOSACCHARIDES

No.	Symbol	Name	Elution time (min)	
			Solvent I[a]	Solvent II[b]
1	Gly	Glycerol	7.0	5.9
2	Rha	Rhamnose	9.4	7.9
3	Rib	Ribose	–	8.1
4	EryOH	Erythritol	10.1	8.6
5	Xyl	Xylose	12.2	10.0
6	RibOH	Ribitol	14.5	11.8
7	XylOH	Xylitol	–	11.9
8	Ara	Arabinose	–	12.0
9	AraOH	Arabinitol	15.5	12.4
10	GlcNAC	N-Acetylglucosamine	18.0	13.4
11	Fru	Fructose	18.1	14.0
12	Man	Mannose	22.4	16.6
13	Glc	Glucose	24.6	18.3
14	ManOH	Mannitol	–	18.8
15	Gal	Galactose	26.1	20.2
16	GalOH	Galactitol	26.2	–
17	Ino	Inositol	–	43.5
18	Glc1-4Glc	Maltose	–	46.9
19	Gal1-4Glc	Lactose	–	56.9
20	Glc1-1Glc	Trehalose	–	59.1

[a]Solvent I: acetonitrile–water, 83.7:16.3 (v/v).
[b]Solvent II: acetonitrile–water, 80:20 (v/v).

rabbits infected with yeasts than in rabbits infected with germ tubes. These data seem to confirm the results obtained by other workers, who detected increased mannose concentrations during the yeasts phase growth in culture without a significant degree of yeast-mycellium transformation [7]. The increase in erythritol levels did not show a significant difference between rabbits infected with germ tube or yeast.

Chromatograms of serum samples from control subjects and patients with systemic candidosis (Fig. 3) showed a significant difference ($P < 0.001$) in mannose concentration. Mannose values ranged from 50 μg/ml in controls and patients without invasive candidosis to 730 μg/ml in patients with disseminated candidosis. The standard deviation from sample to sample was less than 10% except for the glucose, which was 20%. The uncontrolled diet of the rabbits may be responsible for the latter value. Glycerol concentration did not show any significant change among the groups studied.

Mannose levels in the sera of healthy subjects have been estimated as 15 μg/

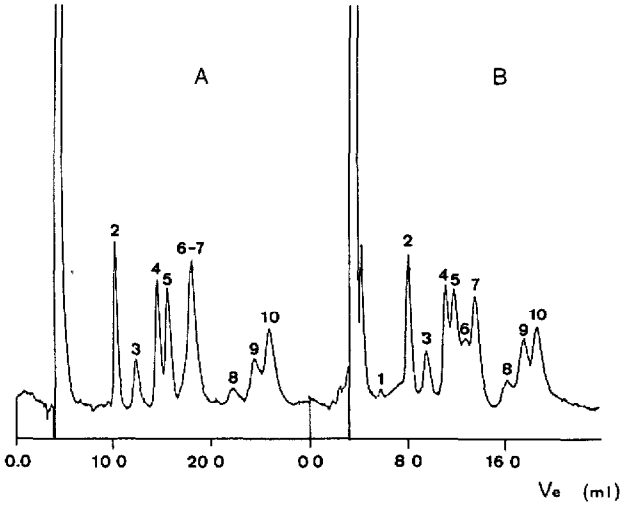

Fig. 1. Chromatograms of reference carbohydrates on a μBondapak carbohydrate analysis column. Flow-rate, 1.0 ml/min; detector, RI at 4×; eluent A, solvent I; eluent B, solvent II. Peaks: 1 = glycerol; 2 = erythritol; 3 = xylose; 4 = ribitol; 5 = arabinitol; 6 = N-acetylglucosamine; 7 = fructose; 8 = mannose; 9 = glucose; 10 = galactitol.

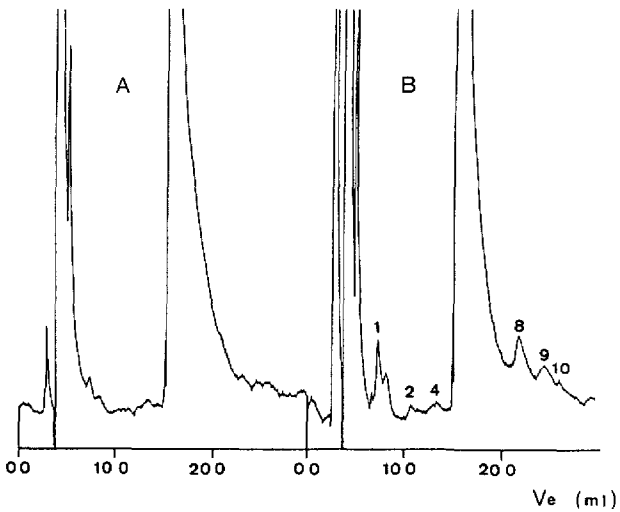

Fig. 2. Chromatograms of rabbit sera (A) before and (B) one day after challenge with $5 \cdot 10^8$ yeast of *C. albicans*. Flow-rate, 1.0 ml/min; detector, RI at 4×; eluent, solvent I. Peaks as in Fig. 1.

ml both by GC and enzyme-based assay [3, 13]. Somewhat higher levels have been reported in sera from rats and from rabbits treated with cortisone [13]. High concentrations of mannose have been found in diabetic patients without systemic candidosis and in patients with aspergillosis [14]. Mannose is pre-

TABLE II

MONOSACHARIDE CONCENTRATIONS IN RABBIT SERA BEFORE AND AFTER INFECTION WITH *CANDIDA ALBICANS*

The S.D. from sample to sample was less than 10%, except for glucose which was 20%. The uncontrolled diet of the rabbits may be responsible for the latter value.

Rabbit serum	Glycerol (μg/ml)	Erythritol (μg/ml)	Ribitol (μg/ml)	Mannose (μg/ml)	Glucose (μg/ml)
Controls	0.005	0	10	0	150
Infected with $5 \cdot 10^8$ yeast	0.170	30	40	1500	150
Infected with 10^8 yeast	0.120	19	70	576	100
Infected with $5 \cdot 10^8$ germ tubes	0.110	10	10	400	250
Infected with 10^8 germ tubes	0.110	60	21	150	200

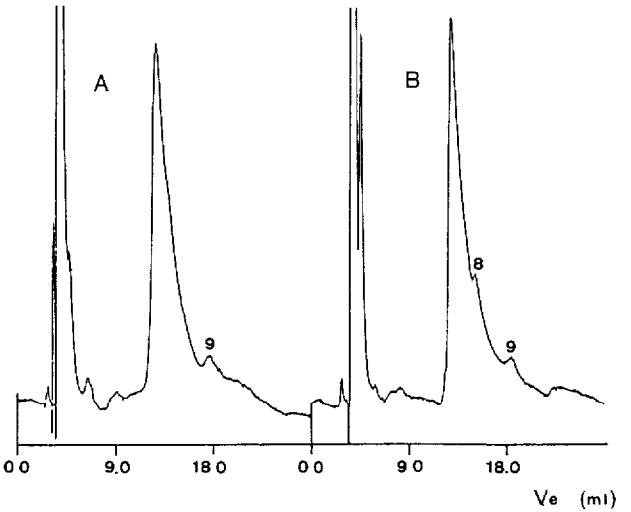

Fig. 3. Chromatograms of human sera from (A) a control subject and (B) a patient with systemic candidiasis. Flow-rate 1.0 ml/min; detector, RI at 4×; eluent, solvent II. Peaks as in Fig. 1.

sumed to derive from the degradation of mannan, a major component of *C. albicans* cell walls [13]. Our data are in agreement with the results published by other authors, who used GC or enzymatic methodology, which suggests that modifications in mannose concentration or its presence may be used as a marker for systemic candidosis [6–8]. The amount of this compound may be related to the extent and severity of the candidal infection. Arabinitol concentrations could not be assessed in our study because its location on the chromatogram was masked by an unidentified peak.

Our results show that HPLC can detect mannose present in serum during the early stages of candidal infection, since HPLC does not require either radiolabelled compounds or the arduous derivatization procedures required by

GC, when anti-*Candida* antibodies are not usually present in sufficient amounts to be detected [1, 3, 15, 16].

ACKNOWLEDGEMENT

We are indebted to Waters Chromatography Division for the carbohydrate analysis column and the use of the differential refractometer.

REFERENCES

1 L. De Repentigny, Eur. J. Clin. Microbiol. Infect. Dis., 8 (1989) 362.
2 J.F. Fischer, R.C. Trincher, J.F. Agel, T.B. Buxton, C.A. Walker, D.H. Johnson, R.E. Cormier, N.H. Chem and J.P Rissing, Am. J. Med. Sci., 290 (1985) 135.
3 F.C. Odds, Candida and Candidosis, Baillere Tindall, London, 2nd ed., 1988, p. 235.
4 M.H. Weiner and W.J. Yount, J. Clin. Invest., 58 (1976) 1045.
5 R.H.K. Eng, H. Chmel and M.J. Buse, J. Infect. Dis., 143 (1981) 677.
6 L. De Repentigny, R.J. Kuykendall, F.W. Chandler, J.R. Broderson and E. Reiss, J. Clin. Microbiol , 19 (1984) 804.
7 T.P. Monson and K.P. Wilkinson, Clin. Microbiol., 14 (1981) 557.
8 R.L. Marrier, E. Millingan and Y.D. Fan, Clin. Microbiol., 16 (1982) 123.
9 G.G. Miller, M.W. Witwer, A.I. Brande and C.E. Davis, J. Clin. Invest., 54 (1974) 1235.
10 S. Honda, Anal. Biochem., 140 (1984) 1.
11 W.M. Blanken, M.L.E. Bergh, P.L. Koppen and D.H. van den Eijnden, Anal. Biochem., 145 (1985) 322.
12 G. Quindos, J. Ponton and R. Cisterna, Eur. J. Clin. Microbiol., 6 (1987) 142.
13 E. Reiss, Molecular Immunology of Mycotic and Actinomycotic Infections, Elsevier, New York, 1986, p. 191.
14 K. Soyama, Clin. Chem., 30 (1984) 293.
15 J.C. Fung, S.T. Donta and R.C. Tilton, J. Clin. Microbiol., 24 (1986) 542.
16 J.W. Bailey, E. Sada, C. Brass and J.E. Bennet, J. Clin. Microbiol., 21 (1985) 749.