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Gas chromatographic determination of D-/L-arabinitol ratio in healthy Polish children

Teresa J. Stradomska*, Zbigniew Mielniczuk[1]

Department of Laboratory Diagnostics, The Children's Memorial Health Institute, Al. Dzieci Polskich 20, 04-736 Warsaw, Poland

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Abstract

The D-/L-arabinitol enantiomers ratio (a marker of disseminated candidiasis of *Candida* species) in urine was determined by gas chromatography (GC) in 198 healthy Polish children ranging in age from 0 to 18 years. The urine samples were dry and trifluoroacetic anhydride (TFAA)-treated. Enantiomers derivatives were separated on a chiral column (β-Dex 120, 60 m×0.25 mm I.D.). A glass ''solid-phase'' injector and electron capture detector (ECD) were used. The ECD response was linear with correlation coefficients 0.999. The limit of detection was 0.02 μmol/l. Good results in terms of reproducibility, accuracy (RSD<10%, bias<6%), and linearity were obtained from real urine samples containing up to 400 μmol/l D-arabinitol. TFA–arabinitol derivatives in biological samples were stable from 1 to 5 days (depending on the arabinitol contents), while TFA–arabinitol standard derivatives were stable for 2 weeks. The identity of D- and L-arabinitol were confirmed by GC–MS analysis. The mean D-/L-arabinitol ratios ranged from 2.48 to 1.65 in the examined groups. The D-/L-arabinitol ratio was found to be exponentially regressive with age. A few cases of diagnosis of disseminated candidiasis by the GC method and confirmed by blood culture are described. The described GC method was also used for monitoring antifungal treatment of patients with disseminated candidiasis.

Keywords: Arabinitol

1. Introduction

Infections caused by disseminated candidiasis have become an increasing therapeutical problem in recent years, mainly in immunocompromised patients, e.g. patients taking high doses of immunosuppressive drugs or undergoing very intensive corticosteroid or multiple antibiotic treatment after organ transplantation, HIV infection, or cancer [1,2]. Very often there are no marked clinical symptoms of infection [1,3]. Additionally, the lack of fast, non-invasive, satisfactory microbiological and serological methods [4,5], is why treatment is started too late in many cases [6,7]. Early and reliable diagnosis is necessary for successful treatment; mortality has been shown to fall from 70 to 30% if antifungal treatment was started before the end of the second day of infection [8]. Because fungal infections pose so many diagnostic and therapeutic difficulties, empirical antifungal therapy is recommended in neutropenic patients with fever not responding to broad-

*Corresponding author. Tel.: +48-22-815-1638; fax: +48-22-815-2456.

E-mail address: maxmz@cobro.org.pl (Z. Mielniczuk).

[1]Present address: Polish Packaging Research and Development Centre, Konstancinska str.11, 02-942 Warsaw, Poland. Tel.: +48-22-842-2011; fax: +48-22-842-23.

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spectrum antibiotic treatment and with suspicion of deep fungal infection [9]. D-arabinitol is a characteristic major metabolite of several pathogenic *Candida* species [10]. *Candida* species account for 79% of all fungal infections [11]; *Candida albicans* is responsible for 75% of fungal infections in neonates [12,13]. Roboz et al. [14] showed that urinary D-/L-arabinitol ratios reflect those in blood, regardless of whether the subject has a deep *Candida* infection or is a healthy individual. Gas chromatography–mass spectrometry (MS) and multi-dimensional GC have been applied to determine of D-/L-arabinitol ratios in clinical specimens by separating trifluoroacetyl derivatives of the arabinitol enantiomers on a chiral GC capillary column [14–19].

The purpose of our study was to establish the D-/L-arabinitol ratio in urine samples taken from healthy Polish children of different age groups using GC as an alternative to the above-mentioned technique. The obtained values would then be the basis of early diagnosis of disseminated candidiasis in major risk groups.

2. Materials and methods

2.1. Chemicals

Trifluoroacetic anhydride was purchased from Sigma (St. Louis, MO, USA), dichloromethane and methanol were acquired from Bayker (Deventer, Holland). *n*-Hexane and toluene were purchased from Merck (Darmstadt, Germany), D- and L-arabinitol from Fluka BioChemika (Buchs, Switzerland). Solvents were of analytical grade and were used without further purification. Diflucan and amphotericin B were from Pfizer.

2.2. GC analysis

A gas chromatograph (HP model 5890 series II) equipped with a laboratory-made glass solid-phase ''falling needle'' injector and electron capture detector (ECD) were used. The fused-silica column (60 m×0.25 mm I.D., 0.25 μm film thickness) was coated with β-cyclodextrin (β-Dex 120, Supelco, Bellefonte, PA, USA). The column was programmed to rise 4 °C/min from 70 to 190 °C. The flow-rate of the helium carrier gas through the column was 2 ml/min. The temperature of the injector was 170 °C and that of the detector was 260 °C. Nitrogen as a make up to the detector was used. A sample (1 μl) was introduced into the tip of the glass needle of the ''solid-phase'' injector.

2.3. GC–MS analysis

The identity of D-arabinitol and L-arabinitol was confirmed by a GC–MS using a Fisons 1000 MS and Carlo-Erba model 8000 GC in the negative chemical ionization mode with ammonia as the reagent gas (10 p.s.i.), and helium as the carrier gas. The ion source temperature was 80 °C. The GC was equipped with the same type of injector and chiral column as in the GC–ECD analysis. The retention times and mass spectra of D- and L-arabinitol in urine sample were identical with authentic derivatized standards [17].The D-arabinitol/L-arabinitol ratio was determined from peak areas. Lack of racemization of arabinitol enantiomers during chromatography was observed.

2.4. Urine collection

The study was carried out on 198 healthy children in various age groups (0–1, 1–3, 3–7, 7–10, 10–18 years). The children were on a conventional diet. Samples (2 ml) of urine were collected and stored at −20 °C before analysis.

Samples of urine were obtained from eight hospitalized high-risk children suspected of disseminated candidiasis. All patients had central venous catheters and were not responding to broad-spectrum antibiotic therapy. In one case, 14 urine samples from an 8-year-old patient with disseminated candidiasis were collected for monitoring of antifungal treatment with diflucan and amphotericin B. In this case the D-/L-arabinitol ratio was calculated over 37 days. The daily dose of diflucan was 8 mg/kg per day, amphotericin B, 0.8 mg/kg per day.

2.5. Preparation of urine samples for GC

Urine samples (1 ml) were filtered through 0.45-μm pore size cellulose acetate filters from Millipore (Molsheim, France) to remove solid material. A

10-μl aliquot of filtrate was transferred to a test tube and evaporated to dryness under a stream of nitrogen at room temperature. Dichloromethane and trifluoroacetic anhydride (200 μl of each) were added, the tubes closed and the samples were heated at 60 °C for 15 min. The reagents were evaporated to dryness and the samples were dissolved in 1 ml of 1% toluene in a mixture of methylene chloride–hexane (1:1, v/v) and subsequently analyzed by GC.

Because in the described method only the ratios of D-/L-arabinitol measured (from peak areas) are the diagnostic marker, use of an internal standard and calculation of the absolute concentration of arabinitol enantiomers is not required [17,23].

3. Results

3.1. GC analysis of urine samples

Separation of D- and L-arabinitol TFA derivatives was achieved after 16 min of analysis. Fig. 1 presents typical chromatograms of a healthy child (D-/L-arabinitol ratio=2.05) and of a patient with disseminated candidiasis (D-/L-arabinitol ratio=5.3).

3.2. Validation of analytical procedure

The limit of detection (LOD) of the GC–ECD system was determined as 0.013 μmol/l for both D- and L-arabinitol TFA derivatives through the whole procedure, with a signal-to-noise ratio of 3.

The method precision was assessed by analyzing two urine samples with low (2.85) and high (6.05) D-/L-arabinitol ratios for 10 consecutive days. Intra-day relative standard deviation (RSD) was 1.24 and 3.0%,whereas inter-day RSD was 2.07 and 5.2%, respectively, for low and high D-/L-arabinitol ratios. GC–ECD instrument precision was determined by repetitive injection of 0.1 μmol/l D-/L-arabinitol standard from the same vial. RSD for the area ratio D-/L-arabinitol for 17 consecutive injections was 1.47%.

The linearity of the ECD was demonstrated: D-arabinitol in amounts of 50, 75, 100, 150, 250 and 500 ng was added to six solutions of L-arabinitol (50 ng) in water (2 ml). Each sample was analyzed in duplicate. The GC–ECD system response was linear

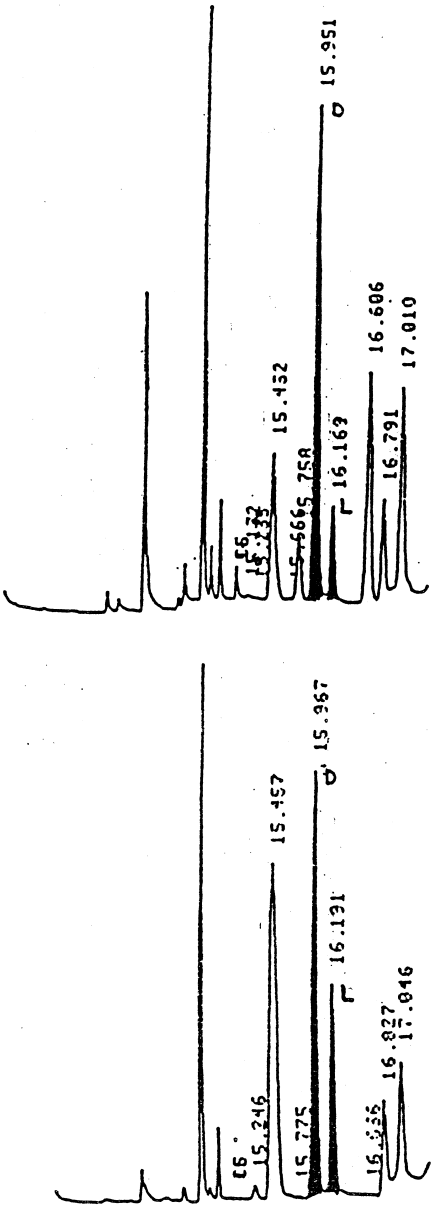

Fig. 1. Chromatogram of D- and L-arabinitol TFA derivatives separated from the urine of a healthy person (bottom) and from a patient with disseminated candidiasis (top).

over the studied range, the equation: $y = 0.021x - 0.11$, coefficient of correlation equal to 0.999.

Linearity of the method was evaluated by analysing for 6 consecutive days blank urine samples with a low D-/L-arabinitol ratio (1.71) and eight samples

of the same urine that had been spiked with a known amount of D-arabinitol (10–500 ng). Precision as relative standard deviations (RSD values expressed as the D-/L-arabinitol ratio) in inter-assay analyses was <10% for all nine concentration ranges (Table 1).

Weighted regression analysis was used to analyze the results; the weight was $1/y^2$.

The correlation coefficient (r) was 0.9968. The linear regression equations were $y = 1.738 + 0.0181x$ for the range from 60 to 400 μmol/l.

The percentage bias between the nominal and measured D-/L-arabinitol ratio in the analyzed samples was below <6%, indicating high accuracy and reproducibility of the method.

3.3. GC–MS analysis

The negative ion-chemical ionization mass spectra of D- and L-arabinitol TFA derivatives were identical. In the high-mass region, ions of m/z ratio 518 (loss of trifluoroacetic acid) and m/z 632 (molecular ion) dominated.

3.4. Stability of the arabinitol TFA derivatives in time

We observed degradation of derivative standards in 1% toluene and a methylene chloride–hexane (1:1, v/v) mixture stored at 5 °C for 4 weeks. Degradation of the D- and L-arabinitol TFA derivatized standards (0.1 μmol/l), after 2 weeks 82% remained, after 3.5 weeks, only 33% (mean of three values). The D- and L-arabinitol TFA derivative standards were stable in excess TFAA at −20 °C for about 5 months.

We observed degradation of D- and L-arabinitol TFA derivatives from urine samples in 1% toluene and a methylene chloride–hexane (1:1, v/v) mixture stored at 5 °C for 2 weeks.

A decrease of the D-/L-ratio (from 2.68 to 1.23) during the period of observation suggests that degradation of the D-enantiomer is much faster than of the L-form.

3.5. Ratios of D-/L-arabinitol in urine of healthy children

The ratios of D-/L-arabinitol enantiomers in the urine samples of 198 healthy children (0–18 years) are given in Table 2. The D-/L-arabinitol ratio (mean±SD) in the 0–1 and 1–3 year-groups were almost the same: 2.48±0.58 and 2.30±0.58. Also the means found for year-groups 3–7 and 7–10 were related, however, about 0.4 lower. The highest average D-/L-arabinitol ratio (2.48) was found in the youngest, 0–1 year-group.

Statistical analysis assuming a proportional error dispension showed exponential regression between the D-/L-arabinitol ratio and age, described by the equation: $y = 2.469 \exp(-0.0310x)$.

Assuming (x+2SD) as the upper normal limit; were calculated the average regression values and upper normal values for age. These values are: 3.5 for children aged 0–1 years; 3.3 for children 1–3; 3.0 for 3–7; 2.7 for 7–10 and 2.4 for children over the age of 10.

Table 1
Inter-assay precision and accuracy of the method

Amount added D-arabinitol [ng]	Mean±SD D-/L-arabinitol ratio	RSD (%) inter-day	Bias (%)
(−) Original samples	1.71±0.030	1.75	–
10	1.90±0.025	1.32	+0.5
25	2.22±0.210	9.45	+4.6
50	2.66±0.200	7.52	−2.3
100	3.45±0.220	6.38	−1.5
150	4.29±0.170	3.96	−2.1
250	6.24±0.250	4.00	+1.3
375	8.29±0.550	6.63	−1.2
500	11.21±0.610	5.44	+5.5

Table 2
Ratio of D-/L-arabinitol in urine of healthy children in different age groups

Groups of age/year	Number of children	D-/L-arabinitol ratio/range	Mean±SD	Normal value[a]
0–1	39	1.47–3.40	2.48±0.58	3.5
1–3	36	1.43–3.34	2.30±0.58	3.3
3–7	42	1.09–3.28	2.08±0.59	3.0
7–10	35	1.08–3.06	1.86±0.45	2.7
10–18	46	0.97–2.46	1.65±0.36	2.4

[a] Upper normal values for age, estimated according to exponential regression.

Table 3
Cases of the patients with disseminated candidiasis

Patient no.	Age (years)	D-/L-arabinitol	Diag. based blood culture
1	3.5	4.24	+ (post mortem)
2	3	4.89	+
3	1	16.90	+
4	2	6.05	+
5	6.5	4.70	+
6	8	4.39	+
7	6	6.70	+
8	4	4.48	+

3.6. Patients with disseminated candidiasis

Table 3 shows examples of diagnosing disseminated candidiasis using the described method. All hospitalized children were considered to be at high risk for invasive candidiasis: four with congenital heart diseases (patients 2, 5, 6, 8), two with abdominal surgery (patients 4, 7), and immunocompromised patients 1 and 3. Urine samples from all of these patients had higher D-/L-arabinitol ratios than healthy children. Diagnosis based on gas chromatography was confirmed by *Candida*-positive blood cultures.

One example of monitoring D-/L-arabinitol ratio in a child at high risk showing time courses for development of candidiasis. A patient, a 2-year-old child with congenital heart disease, was undergoing very invasive treatment in the intensive care unit. Due to this, the monitoring of D-/L-arabinitol ratio in urine was started. The results has been shown in Fig. 2. Initial D-/L-arabinitol ratio was 2.42, after 12 days increasing to 4.24. Than antifungal treatment (Diflucan) was applied with a good results.

3.6.1. Case report: monitoring anti-fungal treatment

A patient (8 years old) was hospitalized for an abdominal operation. Initially, the patient's D-/L-arabinitol ratio was 5.3 (Fig. 1a). Diflucan treatment was instituted for this reason. There was no effect after 11 days of treatment, the arabinitol ratio rose to 6.3 (a *Candida*-positive blood culture confirmed the infection) and another anti-fungal agent Amphotericin B, was started. The D:L ratio dropped dramatically to a normal value (2.5) after 15 days. Treatment was stopped after 12 more days when the D-/L-arabinitol ratio was established between 2 and 3 (Fig. 3).

4. Discussion

In the studied groups of healthy children we observed an exponential relation between the urinary D-/L-arabinitol ratio and age. The mean value in the particular age groups decreased with age. Analysis of the upper normal values (Table 2) led us to adopt a three-stage clinical diagnostic standard for the D-/L-

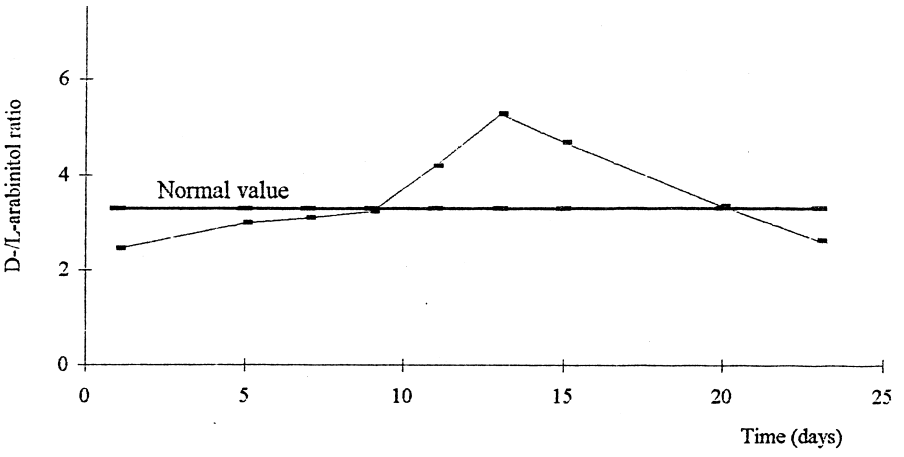

Fig. 2. Monitoring of D-/L-arabinitol ratio in urine in a child at high-risk showing time-courses for development of candidiasis.

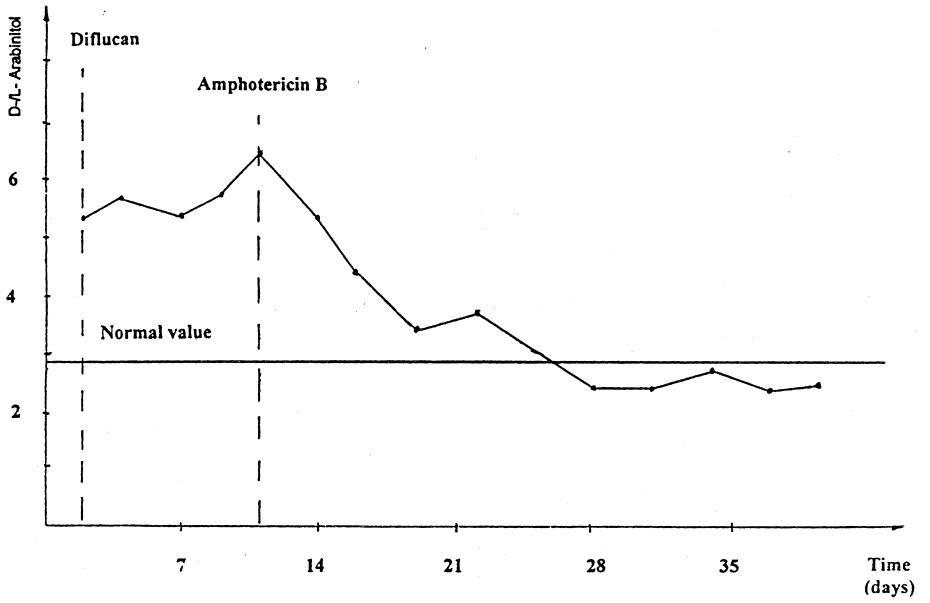

Fig. 3. Monitoring of antifungal treatment of a patient with disseminated candidiasis; results expressed as the D-/L-arabinitol ratio over time (days).

arabinitol ratio in the urine of healthy children. These values are: 3.3 for children aged 0–3 years; 3.0 for children 3–10; and 2.7 for children over the age of 10. This range will be the basis for early diagnosis of disseminated candidiasis of patients in risk groups.

The calculated (mean±SD) value for all of the healthy studied children (198) aged 0–18 years was 2.1±0.56 and is comparable with the results of control groups of non-hospitalized children reported by other authors (Larsson [17]: in five subjects the mean D-/L-arabinitol ratio was 2.0; Christensson [21]: 56 subjects (aged 1–15 years) with a mean of 2.0 (±0.6)). The mean D-/L-arabinitol ratio in our oldest age group (10–18 years) was, however, lower than in control groups of adults in the studies by Roboz [14] (13 subjects with a mean of 1.75 (±0.4) and Lehtonen [23] (50 subjects with a mean of 1.95(±0.34)). The opposite should rather be expected.

It may be surmised that the highest ratio of D-/L-arabinitol in the youngest age group (0–1 years) is a reflection of these children's immature immune systems, elevated *Candida* colonization of mucosae and suppression of gastrointestinal tract bacteria.

The case of a 2-year-old child at high risk (Fig. 2) showed that monitoring D-/L-arabinitol ratio was a very good approach and made it possible to recognize the moment of fungal infection. Antifungal treatment in this case started just in time.

The example of another patient (Fig. 3) showed that monitoring D-/L-arabinitol ratio was also very useful and helped to take appropriate antifungal drug. From this example one can be convinced that choice of the proper antifungal drug should be taken individually in each case.The cases of the patients at high risk for invasive candidiasis (Table 3) showed that in all cases D-/L-arabinitol ratio was higher than in healthy children (from 4.24 up to 16.9).We can believe that monitoring of high-risk children give us a fast diagnosis and suitable early started antifungal treatment. Monitoring therapy makes optimum conditions to control the effects of the treatment and selection of the adequate drugs. Taking into consideration that an elevated D-/L-arabinitol level frequently precedes a positive culture by several days [21–23] it is ideal for early diagnosis in children from high-risk groups as well as for monitoring treatment.

Opportunistic microorganisms are responsible for

most systemic fungal infections and fungemias [20]. The proposed method eliminates the risk of introducing of external etiologic factor (culture-false positive results). It should be a method of choice in small children with still immature immune systems as well as in children undergoing immunosuppression, in which serological diagnostic methods for fungal infection are very often of little value.

The above method is rapid (the full analytical procedure takes 2 h), requires a small amount of material (10 μl of urine) and is non-invasive. Study of the stability of the material over time to assess possible changes in the relative amounts of D- and L-arabinitol enantiomers in a given sample, showed that the use of both improperly stored derivatized standards and samples may lead to false-negative results. TFA–arabinitol standard derivatives in a methylene chloride–hexane can be stored at a temperature of 5 °C only for 2 weeks.

This study, done on a wide population, makes it possible to determine a reliable reference value of the D-/L-arabinitol ratio in the urine of healthy children. Regular monitoring of the D-/L-arabinitol ratios in the urine of high-risk children can be promising, noninvasive method in the diagnosis of fungal infections—a rise in these values may be a sign that antifungal chemotherapy should be instituted, makes possible to avoid an empirical administration of antifungal drugs presently practiced in patients from risk groups.

References

[1] G. Bodey, Candidiasis, Pathogenesis, Diagnosis and Treatment, Raven Press, New York, 1993, p. 279.
[2] J. Vazques, V. Sanchez, C. Dmuchowski, L. Dembry, J. Sobel, M. Zervos, J. Infect. Dis. 168 (1993) 195.
[3] B. Dupont, in: D.W. Warnock, M.D. Richardson (Eds.), Fungal Infection in the Compromised Patient, Wiley, New York, 1991, p. 56.
[4] M. Thaler, B. Pastakia, T.H. Shawker, T.O. Leary, P.A. Pizzo, Ann. Intern. Med. 108 (1988) 88.
[5] L. de Repentigny, Clin. Infect. Dis. 14 (1992) 21.
[6] R. Meunier, A. Aoun, N. Bitar, Clin. Infect. Dis. 14 (Suppl. 1) (1992) 120.
[7] S.B. Wey, M. Mori, M.A. Pfaller, R.F. Woolson, R.P. Wenzel, Arch. Intern. Med. 148 (1988) 2642.
[8] C.H. Debusk, R. Daoud, M.C. Thirumoorthi, M.C. Wilson, R. Khatib, Scand. J. Infect. Dis. 26 (1994) 697.
[9] J. Walsh, J. Lee, J. Lecionnes, M. Rubin, K. Butler, P. Francis, M. Weinberger, E. Roilides, D. Marshall, J. Gress, P. Pizzo, Rev. Infect. Dis. 13 (1991) 496.
[10] E.M. Bernard, K.J. Christiansen, S. Tsang, T.E. Kiehn, D. Armstrong, J. Clin. Microbiol. 14 (1981) 189.
[11] W. Jarvis, Nosocomial infection in the US National Nosocomial Infections Surveillance System, January 1980–April 1990, in: Proceedings of the 3rd International Conference on Nosocomial Infections, 1990.
[12] P.A. Hughes, M.L. Lepow, H.R. Hill, in: G.P. Bodey (Ed.), Candidiasis. Pathogenesis, Diagnosis and Treatment, Raven Press, New York, 1993, p. 261.
[13] F. Meunier, in: News, Cambridge Medical Publication, 1995, p. 2.
[14] J. Roboz, E. Nieves, J.F. Holland, J. Chromatogr. 500 (1990) 413.
[15] J. Roboz, R.N. Katz, J. Chromatogr. 575 (1992) 281.
[16] B. Wong, M. Castellanos, J. Chromatogr. 495 (1989) 21.
[17] L. Larsson, C. Pehrson, T. Wiebe, B. Christensson, J. Clin. Microbiol. 32 (1994) 1855.
[18] E. Chryssanthou, L. Klingspor, J. Tollemar, B. Petrini, L. Larsson, B. Christensson, O. Ringden, Mycoses 42 (1999) 239.
[19] B. Christensson, G. Sigmundsdottir, L. Larsson, Med. Mycol. 37 (1999) 397.
[20] M. Richardson, D.W. Warnock, Fungal Infection. Diagnosis and Management, Blackwell, Oxford, 1993.
[21] B. Christensson, T. Wiebe, C. Pehrson, L. Larsson, J. Clin. Microbiol. 35 (1997) 636.
[22] J. Roboz, Chirality 6 (1994) 51.
[23] L. Lehtonen, V. Anttila, T. Ruutu, J. Salonen, J. Nikoskelainen, E. Eerola, P. Ruutu, J. Clin. Microbiol. 34 (1996) 2175.