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COMPARISON OF METHODS FOR THE DETERMINATION OF β-ASPARTYLGLYCINE IN FECAL SUPERNATANTS OF LEUKEMIC PATIENTS TREATED WITH ANTIMICROBIAL AGENTS

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SUMMARY

β-Aspartylglycine is an indicator of the absence of bacterial enzymatic activity in the intestinal tract. This study describes and compares four methods — (1) dansylation with thin-layer chromatography, (2) ion-exchange chromatography, (3) thin-layer electrophoresis, (4) high-voltage paper electrophoresis — to determine the concentration of β-aspartylglycine in fecal supernatants of leukemic patients treated with antimicrobial agents.

INTRODUCTION

β-Aspartylglycine was found to be present in cecal contents of germ-free mice and mice treated with certain antibiotics, whereas it was not present in the intestinal tract of control animals [1]. Later on it was shown that this dipeptide was also present in the cecal contents of other germ-free animal species such as rats, lambs and piglets, and in the feces of totally decontaminated leukemic patients [2]. Subsequently, it was found that after association of germ-free mice with an increasing number of different strains of anaerobic bacteria, the concentration of β-aspartylglycine decreased gradually, reaching undetectable levels after association with 50—60 strains of bacteria [3]. The bacterial flora in the gastrointestinal tract is constantly producing enzymes which interact with host enzymes. It was shown that β-aspartylglycine could only be degraded by a bacterial enzyme originating from a number of strains of bacteria [4].

The bacterial flora which is normally present in the gastrointestinal tract protects the host against potentially pathogenic microorganisms from the en-

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vironment [5]. Since colonization and eventually infection occurs much more easily in germ-free animals and animals treated with certain antibiotics [6], it was decided to use β-aspartylglycine as an indicator of the bacterial infection risk during bacterial decontamination of leukemic patients [6, 7]. Results of these investigations will be published elsewhere. The present study describes and compares four methods to determine the concentration or the presence of β-aspartylglycine in feces.

EXPERIMENTAL

Reference solution of β-aspartylglycine

Different amounts (1—20 μl) of 15% dry weight fecal supernatants from germ-free mice were used as reference solutions to determine the relative concentration of β-aspartylglycine in fecal supernatants from patients. A 15% dry weight fecal supernatant from germ-free mice contains 0.8 μmol of β-aspartylglycine per ml.

Commercially available β-aspartylglycine can also be used as a reference solution, but the following has to be taken into account. Commercial β-aspartylglycine (Serva, Heidelberg, G.F.R.; Sigma, St. Louis, MO, U.S.A.) as a powder does not contain any β-aspartylglycine. It consists of the cyclized α, β-form. In solution it slowly converts to the β-form. To prepare a reference solution, the cyclized α,β-aspartylglycine was dissolved in water and incubated for 30 days at 37°C. The resulting mixture of β-aspartylglycine, α,β-aspartylglycine and a small amount of α-aspartylglycine can be used as reference solution. In some cases, however, this β-aspartylglycine-containing solution was further purified by preparative high-voltage paper electrophoresis at pH 3.5 [1]. Different dilutions of a solution of this preparation were subjected to high-voltage paper electrophoresis and compared with the reference solution obtained from 15% dry weight fecal supernatants from germ-free mice. The concentration of the β-aspartylglycine solution was determined by amino acid analysis (Kontron Liquimat III) of an acid-hydrolyzed sample using norleucine as an internal standard.

Dansylation and thin-layer chromatography

To 25 μl of fecal supernatant (25%, w/v) were added 5 μl of 0.1 *M* sodium hydroxide and 30 μl of Dns-Cl (1-dimethylaminonaphthalene-5-sulfonyl chloride) solution (5 mg/ml of acetone). The mixture was incubated for 30 min at 37°C. After centrifugation 50 μl of the supernatant were added to 3 ml of 0.01 *M* acetic acid and subsequently a small amount of Dowex 50 (H^+) equilibrated with 0.01 *M* acetic acid [9]. This suspension was washed three times with 6 ml of 0.01 *M* acetic acid to remove Dns-OH. Finally, the suspension was washed with 2 ml of ammonia—acetone (1:1, v/v) to remove the Dns-amino acids from the resin. The ammonia—acetone wash was freeze-dried and dissolved in 5 μl of ethanol. The Dns-amino acid mixture was applied to both sides of a polyamide layer (5 × 5 cm) (Schleicher and Schüll, Dassel, G.F.R.). To one side also a Dns-β-aspartylglycine reference solution was added. The polyamide sheets were chromatographed in formic acid—water (15:500, v/v) and subsequently perpendicular to the direction of the first solvent in ethyl

acetate—formic acid—methanol (50:2.5:2.5, v/v). The layers were examined under ultraviolet light.

Ion-exchange chromatography

To 50 μl of 25% (w/v) fecal supernatant 500 μl of methanol were added. After mixing and centrifugation 400 μl of the supernatant were removed and dried at 60°C in a stream of nitrogen. The residue was dissolved in 20 μl of the sample buffer for amino acid analysis; 5 μl (the equivalent of 9 μl of fecal supernatant) were applied to the Kontron Liquimat III amino acid analyzer.

Thin-layer electrophoresis

To 40 μl of fecal supernatant (25%, w/v) were added 500 μl of methanol. After mixing and centrifugation the supernatant was dried under a stream of nitrogen at 60°C. The residue was dissolved in 15 μl of the electrophoresis buffer pH 3.5 (see below). Of this solution 2—10 μl were applied as a line 1 cm long at the center of a 20 × 20 cm cellulose MN 300 layer (Polygram cel 300; Macherey-Nagel, Düren, G.F.R.). Electrophoresis was performed at 800 V for 30 min in a home-built apparatus with liquid cooling as described by Whittaker and Moss [10]. After staining with 0.2% ninhydrin in ethanol and drying with a hair-dryer, the layer was heated for 5 min at 110°C, during which the blue to greyish color of β-aspartylglycine may become visible.

High-voltage paper electrophoresis

An 80 μl aliquot of a 25% (w/v) fecal supernatant was applied to Whatman 3 MM chromatography paper (46 × 57 cm) 18 cm from the edge of the paper to be immersed in the anode buffer compartment. A reference solution was applied containing lysine, glycine, glutamic acid, aspartic acid, β-aspartylglycine (see section on reference solution of β-aspartylglycine) and Xylene cyanol FF. The blue-colored dye Xylene cyanol FF (BDH, Poole, Great Britain) migrates slightly ahead of β-aspartylglycine to the anode. High-voltage paper electrophoresis was performed at pH 3.5 (pyridine—acetic acid—water, 1:10:89, v/v) for 1 h at 3000 V in a home-built apparatus similar to the commercially available Savant Model LT48 (Savant, Hicksville, NY, U.S.A.). After elecrtrophoresis the paper was dried at 70—80°C for 10 min and dipped in 0.2% ninhydrin in ethanol. Heating at 70—80°C resulted in purple spots for most of the peptide-like material, except for β-aspartylglycine which shows up greyish. Additional heating at 120—150°C for 10 min gives a clear blue color for β-aspartylglycine. The concentration of β-aspartylglycine in the fecal supernatants was determined by visual comparison with the intensity of the color of various concentrations of β-aspartylglycine reference solutions.

RESULTS

Cyclized α,β-aspartylglycine converted in solution to the more stable β-form for about 70% in 30 days. This solution was used as a reference solution next to a 15% dry weight fecal supernatant from germ-free mice which contained 0.8 μmol of β-aspartylglycine per ml. The concentration of free amino acids varied from 1.4 to 12.8 μmol/l.

Dansylation and thin-layer chromatography

Thin-layer chromatography of a solution containing Dns-α-aspartylglycine, Dns-α,β-aspartylglycine, Dns-β-aspartylglycine and some other Dns-amino acids showed that the different Dns-aspartylglycines were very well separated from the Dns-amino acids (Fig. 1A). To determine the presence of β-aspartylglycine in fecal supernatants, samples have to be pretreated with acetone to remove

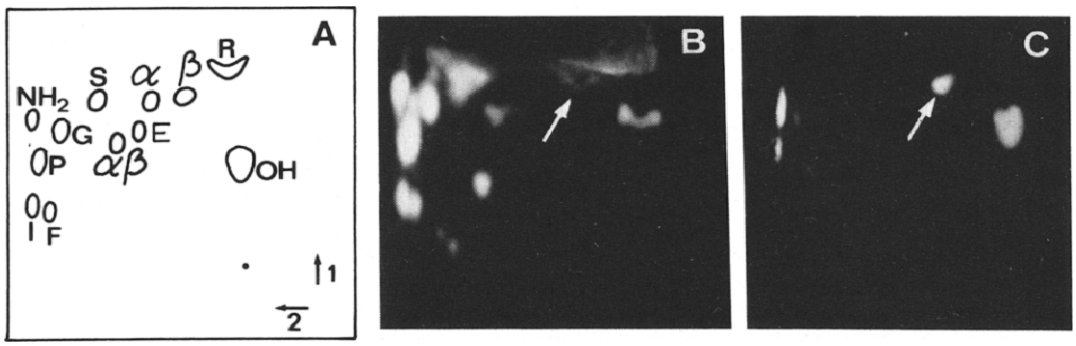

Fig. 1. Thin-layer chromatography on polyamide layers. (A) Dansylated amino acids and peptides: R = arginine, S = serine, E = glutamic acid, G = glycine, P = proline, I = isoleucine, F = phenylalanine, β = β-aspartylglycine, α = α-aspartylglycine; αβ = α,β-aspartylglycine, Dns-NH_2 and Dns-OH are also indicated. (B) Dansylated fecal supernatant containing β-aspartylglycine (arrow). (C) Dansylated β-aspartylglycine (arrow). Solvent in the first dimension was formic acid—water (3:100) and in the second dimension ethyl acetate—formic acid—methanol (20:1:1). The layers were examined under ultraviolet light.

TABLE I

PRESENCE OR CONCENTRATION (μ*M*) OF β-ASPARTYLGLYCINE IN FECAL SUPERNATANTS FROM LEUKEMIC PATIENTS TREATED WITH ANTIBIOTICS

Sample	Dansylation*	Ion-exchange chromatography**	Thin-layer electrophoresis*	High-voltage paper electrophoresis***
1	+	320	++	320
2	+	240	++	300
3	ND†	210	ND	200
4	+	ND	++	180
5	±	210	+	140
6	±	170	+	120
7	–	ND	+	100
8	ND	180	ND	85
9	+	65	–	35
10	–	80	–	18
11	ND	55	ND	10

*Semi-quantitative measurement from – to ++.

**Amount based on peak height; the concentrations were based on the assumption that sample 1 (Fig. 2 bottom) had the same concentration as determined by high-voltage paper electrophoresis.

***Concentration (μ*M*) from semi-quantitative measurements (above 50 μ*M*: ±10%; below 50 μ*M*: ±20%).

† ND = not done.

proteins and with Dowex 50 to remove most of the Dns-OH formed during the dansylation reaction. Examples of thin-layer chromatography of a dansylated fecal supernatant and a reference solution are shown in Figs. 1B and C, respectively. The results are shown in Table I.

Ion-exchange chromatography

After removal of protein by methanol precipitation, fecal supernatants were subjected to cation-exchange chromatography on an amino acid analyzer using a short program intended for analysis of acid-hydrolyzed proteins. Part of the analysis of two fecal supernatants is shown in Fig. 2. Pure β-aspartylglycine showed only one peak (see Fig. 2, β). All fecal supernatants showed peaks at almost the same elution position as β-aspartylglycine whether they contained

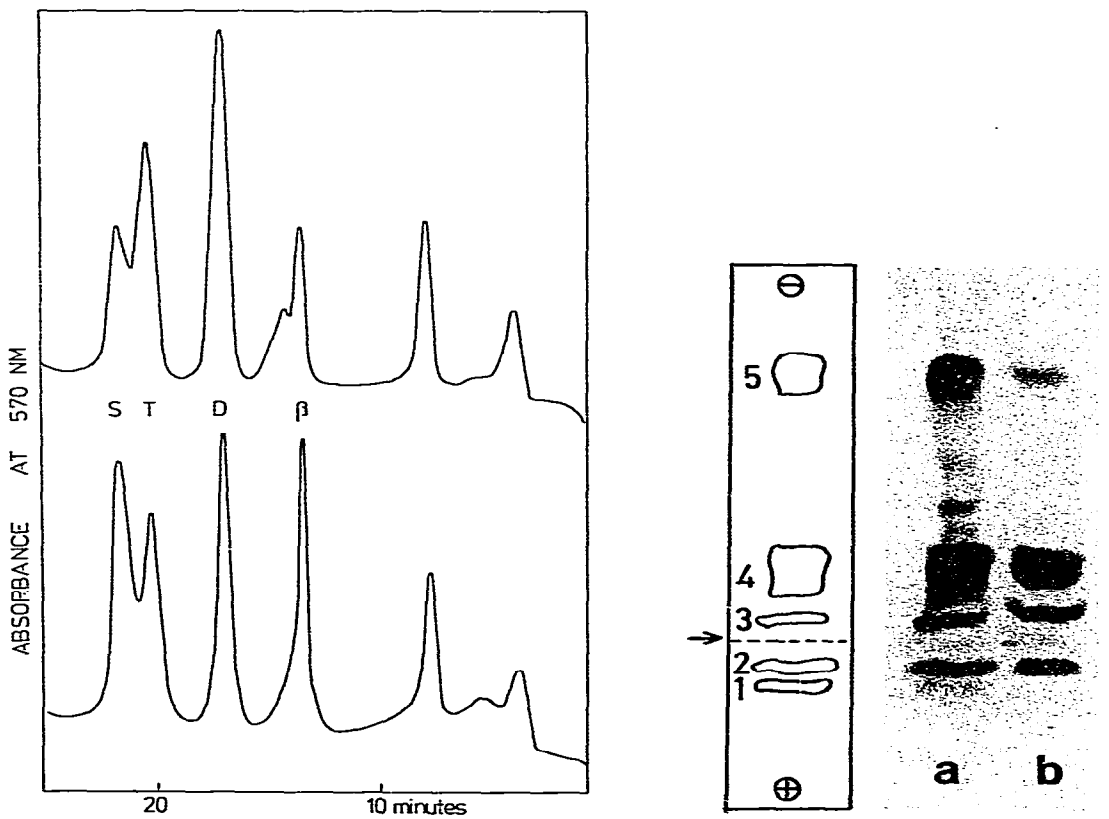

Fig. 2. Ion-exchange chromatography of fecal supernatants from leukemic patients treated with antibiotics. S = Serine, T = threonine, D = aspartic acid, β = β-aspartylglycine. Supernatants obtained after methanol precipitation were applied to an amino acid analyzer. Top: pattern obtained from feces of a patient after 17 days of oral treatment with cephradine (6 g/day). Bottom: pattern from feces of a totally decontaminated patient.

Fig. 3. Thin-layer electrophoresis of fecal supernatants from leukemic patients at 800 V for 30 min. 1 = β-Aspartylglycine, 2 = aspartic acid, 3 = glutamic acid, 4 = neutral amino acids, 5 = basic amino acids. (a) A fecal supernatant containing β-aspartylglycine; (b) a sample containing no β-aspartylglycine. The application line is indicated by an arrow.

β-aspartylglycine or not. These peaks interfered with the accurate determination of β-aspartylglycine. The results based on peak height are shown in Table I.

Thin-layer electrophoresis

Electrophoresis of fecal supernatants without pretreatment often resulted in distorted amino acid and peptide spots and uneven migration. These problems were overcome by removal of the proteins present in the samples by methanol precipitation. An example of thin-layer electrophoresis of fecal supernatants from leukemic patients treated with antibiotics is shown in Fig. 3. The results are shown in Table I.

High-voltage paper electrophoresis

Fecal supernatants were applied to Whatman 3 MM chromatography paper without any pretreatment. Examples of electrophoresis of patient fecal samples containing no β-aspartylglycine, a reference solution of β-aspartylglycine and different amounts of a fecal supernatant from germ-free mice are shown in Fig. 4. The electrophoresis results are summarized in Table I.

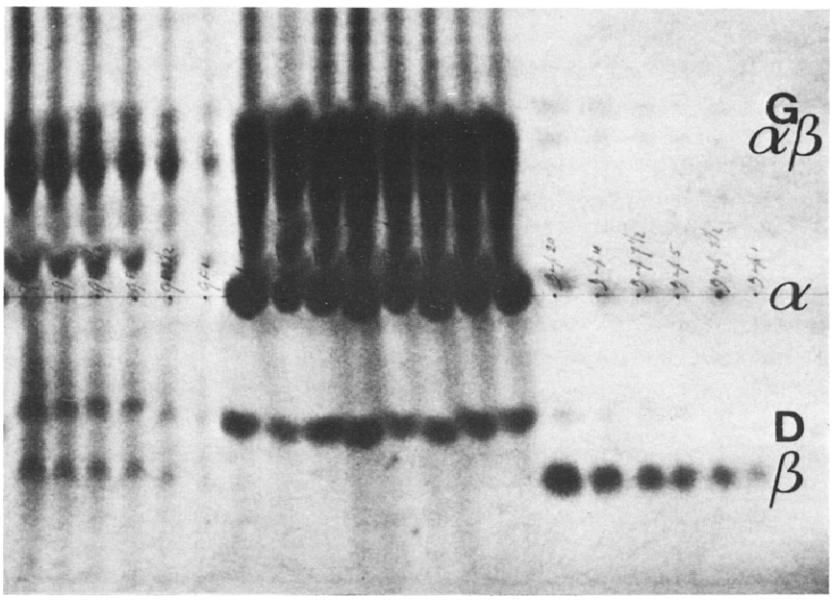

Fig. 4. High-voltage paper electrophoresis at pH 3.5 for 1 h at 3000 V. From left to right: 20, 10, 7.5, 5, 2.5 and 1 μl of a 15% dry weight fecal supernatant from germ-free mice; eight fecal supernatants containing no β-aspartylglycine; a reference solution of β-aspartylglycine (β) containing small amounts of α-aspartylglycine (α), aspartic acid (D), glycine (G) and α,β-aspartylglycine (αβ).

DISCUSSION

In Table I the results obtained with the four methods are compared. Generally there is a good agreement between the methods used. Dansylation is the less sensitive method to determine β-aspartylglycine. Since the other amino acids

are generally present in much higher concentration, the presence of Dns-β-aspartylglycine can only be detected in samples with relatively high concentrations of β-aspartylglycine. Furthermore, the shape and size of the Dns-arginine spot may give problems in distinguishing the presence of β-aspartylglycine.

Thin-layer electrophoresis using the liquid-cooled home-built apparatus described by Whittaker and Moss [10] turned out to be a reliable method to determine the presence of β-aspartylglycine. However, only a limited amount of sample could be applied to the cellulose thin-layer. Furthermore, pretreatment of the supernatants with methanol to remove protein is very often necessary. Although this treatment generally did not have any effect on the recovery of β-aspartylglycine, in a few cases a 25% loss of β-aspartylglycine was observed.

Since a fecal supernatant is rather dirty, analysis on an amino acid analyzer is preferably also preceded by a methanol precipitation step. β-Aspartylglycine can be separated very well from the common amino acids but fecal supernatants appeared to contain substances which eluted at almost the same position as β-aspartylglycine. This makes accurate determination of the concentration impossible, especially at concentrations of β-aspartylglycine of less than 100 μM. Because of this interference, concentrations calculated from the height of the peak at the elution position of β-aspartylglycine are most probably higher than the values obtained with high-voltage paper electrophoresis. Furthermore, other β-aspartylpeptides, i.e. β-Asp-Ala, β-Asp-Ser and β-Asp-Gln, were shown to be present in feces of decontaminated patients [11]. With the short elution program intended for routine analysis of acid-hydrolyzed protein samples, these peptides elute at the same position as β-aspartylglycine, although β-aspartylglycine is most predominantly present. With other gradients however these peptides might be separated [12—14]. The aforementioned factors will result in an over-estimation of the β-aspartylglycine concentration when determined by ion-exchange chromatography (see Table I, column 3). Despite these difficulties, ion-exchange chromatography provides a simple way of determining the presence of β-aspartylglycine in concentrations above 100 μM.

High-voltage paper electrophoresis is the method of choice to determine the concentration of β-aspartylglycine in fecal supernatants. Twenty to twenty-five fecal supernatants can be applied directly, without any pretreatment, to one piece of chromatography paper and subjected to electrophoresis. β-Aspartylglycine is more acidic than aspartic acid at pH 3.5 and because of its blue color after staining with ninhydrin and subsequent heating it can be easily distinguished from the purple spots of other peptide-like substances.

Table I, which summarizes the results, shows that β-aspartylglycine can be detected with all four methods in fecal samples containing more than 0.1 μmol/ml. The most accurate determination of lower concentrations (0.01—0.1 μmol/ml) can only be done with high-voltage paper electrophoresis. The latter method has been applied to monitor disturbance of the bacterial flora of immunocompromised patients who are at high risk of infection. These patients received a preventive treatment with antimicrobial drugs that only removed the aerobic potentially pathogenic microorganisms [7, 8], leaving the major part of the bacterial flora in the gastrointestinal tract intact. β-Aspartylglycine was found in 7% of 793 samples investigated. After additional treatment

with orally administered cephradine, β-aspartylglycine was found in 42% of 81 samples investigated. This indicated that a substantial part of the protective bacterial flora was removed.

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