Detection of Aspartylglycosaminuria by Gas–Liquid Chromatography

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I describe a rapid gas-chromatographic method for specific detection of the lysosomal storage disease aspartylglycosaminuria, based on the identification of the major storage compound, 2-acetamido-1-N(4'-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine (GlcNAc-Asn) in the urine of affected individuals. A 50-µl sample of urine is methylated without prior purification; the methylation products are then analyzed by gas–liquid chromatography. Under these conditions a diagnostic GlcNAc-Asn peak can be seen in the urine of patients with aspartylglycosaminuria, but not in the urine of control subjects or patients with related storage diseases.

Additional Keyphrases: storage disease • urine • lysosomal enzymes • heritable disorders

Aspartylglycosaminuria (AGU), an inborn error of glycoprotein catabolism, is characterized by the accumulation of glycosaminoglycans in tissues and urine (1–5). Clinical characteristics include progressive mental retardation, connective tissue abnormalities, and recurrent infections (6, 7). Most cases (about 130) have been diagnosed in Finland, but non-Finnish cases are also known (8).

Laboratory diagnosis of AGU is usually based on the demonstration of the main storage compound, 2-acetamido-1-N-(4'-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine (GlcNAc-Asn) in urine or tissue samples by chromatographic methods (1–3, 9–11). Alternatively, the demonstration of decreased 1-aspartamido-β-N-acetylglucosamine amidohydrolase activity (EC 3.5.1.26) in leukocytes or cultured fibroblasts can be used for diagnosis (12, 13). Combined gas-chromatographic/mass-spectrometric analysis of urine provides definite structural identification of GlcNAc-Asn (10), but this method is not suitable for routine laboratory diagnosis. I describe here a simplified gas-chromatographic procedure for the rapid, specific detection of increased amounts of GlcNAc-Asn in urine, for use as a diagnostic test for AGU.

Materials and Methods

Patients and normal subjects. Forty patients, ages 3 to 44 years, with confirmed AGU were studied. A total of 70 urine samples were collected from these patients and frozen until processed. Urine samples were also obtained from patients with confirmed mannosidosis, fucosidosis, Salla disease, GM1-gangliosidosis, metachromatic leukodystrophy, and Fabry’s disease. Urine samples from 20 healthy subjects, ages six to 50 years, served as control samples. Patients and controls received food ad libitum during the sampling period.

Reference compounds. GlcNAc-Asn was obtained from Vega-Fox Biochemicals. Melibiose used as an internal standard was prepared from melibiose (Eastman Organic Chemicals, Rochester, NY 14650) by borohydride reduction (14).

Gas–liquid chromatography. A gas chromatograph with hydrogen flame ionization detectors (Model 900; Perkin-Elmer Corp., Norwalk, CT 06856) was used. Glass columns, 2 m x 3 mm (i.d.), were packed with 2.2% SE-30 on Gas Chrom Q (Applied Science Lab., State College, PA 16801). Nitrogen was used as carrier gas. The columns were maintained at 250 °C.

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Mass spectrometry. I used a Mat CH-7 mass spectrometer equipped with a Spectro System 100 MS data-processing system (both from Varian Associates, Palo Alto, CA 94303). Ionization current was 300 μA, ionization potential 70 eV.

Methylation of urine samples. Methylation (15–17) was performed in Teflon-lined 14 × 100 mm screw-cap tubes at room temperature. The tubes were flushed with nitrogen at each step of the procedure. Methylsulfinylcarbanion was prepared by dissolving, during 30 min at 60 °C, 3 mol of potassium tert-butylate (Merck, Schuchardt) per liter of dimethyl sulfoxide; the base concentration of the reagent was 1.9 mol/L. This reagent was stored in a desiccator at 4 °C; before use, it was thawed at room temperature and centrifuged. Samples (50 μL of urine, reference GlcNAc-Asn, and blank with or without added melibiotol as internal standard) were dried under nitrogen, dissolved in 100 μL of dimethyl sulfoxide, and sonicated. One hundred microliters of methylsulfinylcarbanion was added to the samples and sonicated for 1 h. The samples were tested for excess base with triphenylmethane (16) and sonicated for another hour, after which 150 μL of methyl iodide was added with cooling. After sonication for a further hour, the samples were diluted with water to 2 mL and partitioned with 2 mL of chloroform. The chloroform was extracted three times with water and dried. The methylation products were dissolved in 50 μL of chloroform.

Results

Figure 1 illustrates typical gas chromatograms obtained by this method. A peak with the retention time of GlcNAc-Asn is clearly seen in urine from AGU patients, whereas chromatograms of control urine and urine from patients with mannosidosis, fucosidosis, Salla disease, GM1-gangliosidosis, Fabry’s disease, or metachromatic leukodystrophy lack the GlcNAc-Asn peak under these conditions. The compound represented by the peak was compared with authentic GlcNAc-Asn by mass spectrometry, and the two were found to be identical.

A total of 70 urine samples from 40 AGU patients were analyzed by this method; all were positive for GlcNAc-Asn.

Discussion

Normal individuals excrete relatively small amounts of GlcNAc-Asn in their urine (18). Mass-fragmentographic analysis has revealed concentrations in the range of 0.3–2.7 mg/L (3). The concentrations of GlcNAc-Asn found in the urine of AGU patients are, however, markedly higher, varying between 110 and 510 mg/L (mean, 300 mg/L), as estimated by quantitative gas chromatography (3).

The sensitivity of the present method was chosen for easy detection of AGU patients with relatively low GlcNAc-Asn output—i.e., about 100 mg/L—but not the normal output of GlcNAc-Asn of healthy individuals. The reliability of the urinary assay of above-normal GlcNAc-Asn excretion is increased by the fact that GlcNAc-Asn output seems to be fairly constant in the same patient on consecutive days (8). Furthermore, increased GlcNAc-Asn excretion seems to precede clinical symptoms of AGU. No other disease is known to be characterized by increased urinary GlcNAc-Asn concentrations. In this study, I tested several related lysosomal storage diseases for increased GlcNAc-Asn excretion and all proved negative.

Obligate heterozygotes (parents of AGU patients) excrete normal amounts of GlcNAc-Asn and cannot be detected with this method. Assessment of 1-aspartamido-β-N-acetylglucosamine amidohydrolase activity in blood or cultured fibroblasts might be valuable for carrier detection (12, 19).

GlcNAc-Asn can be measured quantitatively by adding melibiotol as an internal standard. However, for quantitative purposes, prior purification of the urine samples by anion-exchange chromatography is more reliable, and is recommended when this screening test for AGU is positive (3). In doubtful cases, mass-spectrometric confirmation of the identity of the emerging peak by comparison with authentic GlcNAc-Asn is recommended. Complete methylation of the urine sample is important in the present method, and the use of triphenylmethane to indicate the activity of dimethylsulfinylcarbanion (16) has been satisfactory for this.

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References

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Urinary Free Catecholamines Determined by Liquid Chromatography–Fluorometry

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Free norepinephrine, epinephrine, and dopamine are determined in urine after purification on a small ion-exchange column and concentration on alumina. The catecholamines are separated by reversed-phase ion-pair chromatography and are quantitated by measuring their native fluorescence ($\lambda_{ex}$ 285 nm, $\lambda_{em}$ 305 nm). The internal standard is dihydroxybenezylamine. Within-day CVs for the compounds ranged from 1.6 to 5.6%; between-day CVs were 6.6 to 9.8%.

Additional Keyphrases: norepinephrine · epinephrine · dopamine · chromatography, reversed-phase ion-pair

Measurement of free urinary catecholamine concentrations is of interest in clinical chemistry and neurochemistry laboratories. Recently, "high-performance" liquid chromatographic (HPLC)6 methods (1–5) have been developed to improve on the sensitivity and specificity of the trihydroxindole-fluorometric procedures. The HPLC-electrochemical (LC-EC) procedures (1–4) involve two-step sample purifications, and the amperometric detection systems have yet to be widely accepted in clinical laboratories. In a recently developed HPLC-fluorometric (LC-F) procedure, Jackman (5, 6) uses a one-step cation-exchange purification and detects the native fluorescence of the compounds. However, because the urine is not concentrated before sample injection (~50 µL of urine equivalent), the ~100-pg detection limit for epinephrine does not allow its precise determination in the low normal range (~1–5 µg/L). We report a method with cation-exchange purification and alumina concentration steps similar to those previously used with an LC-EC method (2). However, after separation by reversed-phase ion-pair chromatography, the catecholamines are determined by measuring their native fluorescence.

Materials and Methods

Reagents. 'Norepinephrine, epinephrine, dopamine, Tris base, and alumina (neutral, grade 1, acid-washed upon receipt) were purchased from Sigma Chemical Co., St. Louis, MO 63178. The internal standard, dihydroxybenzylamine (DHBA), was purchased from Aldrich Chemical Co., Milwaukee, WI 53233. Sodium octane sulfonate was obtained from Eastman Organic Chemicals, Rochester, NY 14650; "distilled in glass" methanol was from Burdick and Jackson Labs., Muskegon, WI 49422. Catecholamine isolation columns6 were from Bio-Rad Laboratories, Richmond, CA 94804; the resin was regenerated after use by a batch method (2). All other chemicals were reagent grade from local suppliers.

Stock solutions of the standards (100 mg/L as free base) were prepared in 100-mL quantities every two months in 10

6 Containing a proprietary packing, apparently of wide size range (~50–250 µm) Bio-Rex 70.