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A sensitive method for 4-hydroxybutyric acid in urine using gas chromatography–mass spectrometry

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Abstract

4-Hydroxybutyric acid (4HB) was analyzed by gas chromatography–mass spectrometry. Under acidified conditions, 4HB is difficult to detect due to lactonization. Using a urine sample containing 0.01 mg creatinine, we performed trimethylsilyl derivatization without extraction, only adding dimethylsuccinic acid as an internal standard and 10 μl of 0.1 N NaOH methanol solution with adequate evaporation. Urine 4HB levels in a patient with 4-hydroxybutyric aciduria was determined to be 1258 mmol/mol Cr (control, 0.28–2.81 mmol/mol Cr) in this method. Direct derivatization of samples without extraction showed good reproducibility and linearity. Only a small sample of urine was required. Alkalinization by NaOH prevented not only lactonization of 4HB, but also loss of the compounds during evaporation.

Keywords: 4-Hydroxybutyric acid; 4-Hydroxybutyric aciduria

1. Introduction

4-Hydroxybutyric acid (4HB) is a metabolite of the neurotransmitter 4-aminobutyric acid (GABA) in the central nervous system. 4-Hydroxybutyric aciduria is caused by a defect of GABA degradative pathway at the level of succinic semialdehyde dehydrogenase, resulting in accumulation of 4HB. The symptoms include mental retardation, hypotonia, ataxia and seizures. One hundred and fifty patients have been diagnosed up to now [1]; We had previously reported the first Japanese patient [2,3].

The urinary organic acid analysis by gas chromatography–mass spectrometry (GC–MS) using conventional solvent extraction after acidified urine samples, showed only a small peak for 4HB, which could be easily missed. Acidification converts 4HB to its cyclic lactonic acid, butyrolactone. Therefore, 4HB is hardly detected in routine urinary organic acid analysis. The analytical methods for the detection of 4HB are conversion of 4HB to butyrolactone at a high temperature and with a strong acid followed by liquid–liquid extraction and detection by GC or GC–MS [4–6]; or 4HB itself is isolated using solid-phase extraction [7,8] or liquid–liquid extrac-

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tion [9–11] and derivatized for GC–MS analysis. We developed a sensitive and simple method for measurement of 4HB, characterized by addition of NaOH, and performed trimethylsilyl derivatization without extraction.

2. Materials and methods

2.1. Reagents

Dimethylsuccinic acid, 4HB sodium salt and urease of Sigma type III from Jack beans (870 000 U/g solid) were obtained from Sigma Chemical Co. (St Louis, MO). Bis(trimethylsilyl)fluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and sodium hydroxide (NaOH) were obtained from Nakarai-Tec Co (Tokyo, Japan).

2.2. Sample preparation

Urine 4HB was measured as follows: to a 1.5-ml microtube, a urine sample equivalent to 0.01 mg creatinine was added with 2 units of urease, and then incubated for 30 min at 37 °C. In the case of a urine sample from a patient, 0.0002 mg creatinine equivalent urine was used because of an excessive amount of 4HB. Next, 1 nmol of dimethylsuccinic acid in 10 μl of methanol as an internal standard and 10 μl of 0.1 N NaOH methanol solution were added. The samples were vortexed and centrifuged at 6000 *g* for 5 min. Then the supernatants of samples were transferred to a 1.5-ml glass vial and completely evaporated under a gentle nitrogen stream at 60 °C. The samples were then derivatized by addition of 60 μl of a mixture (100:1 v/v) of BSTFA and TMCS, and allowed to react for 30 min at 80 °C. The reaction mixtures were vortexed and centrifuged at 300 *g* for 5 min. The supernatant of the reaction mixtures was subjected to GC–MS analysis.

2.3. GC–MS analysis

A capillary GC–MS system, Shimadzu model QP5050A (Shimadzu Co., Ltd, Kyoto, Japan), equipped with a Class 5000 data processing system, was used. The capillary column was a fused-silica DB-5 one (30 m×0.25 mm I.D.) with a 0.25-μm film thickness of 5% phenylmethyl silicone (J and W, Folsom, CA). Mass spectra were obtained by electron impact ionization in the selective ion monitoring (SIM) mode at the scan rate of 0.3 s/cycle. The temperature program was started at 60 °C with initial holding for 2 min, increased at a rate of 10 °C/min to 180 °C, then increased to a final temperature of 280 °C at a rate of 35 °C/min with holding for 4 min. Temperatures of the injection port and transfer line were both 280 °C. The flow-rate of the helium carrier was 1.6 ml/min and the linear velocity was 68.9 m/s. One microliter of the final derivatized aliquot was injected onto the GC–MS in splitless mode.

The methylene units or retention indices to hydrocarbons were 12.40 for 4HB and 12.50 for dimethylsuccinic acid. Selected ions used in SIM mode were m/z 117 and 233 for 4HB, and m/z 231 and 275 for dimethylsuccinic acid. For quantitation, calculation of the relative peak areas (%) was made between the quantitative ions of 4HB and those of the internal standards (m/z 233)/(m/z 231). For peak identification and quantitation we used an automated GC–MS analysis system previously developed [12].

Standard curves for the method obtained were as follows: a fixed amount of internal standard, 1 nmol of dimethylsuccinic acid, and various amounts of the 4HB methanol solution with 0.05, 0.1, 0.2, 0.5, and 1 nmol were analyzed.

Reproducibility was determined by analyzing 1 nmol standard solution of 4HB and 0.01 mg creatinine equivalent urine from a healthy child in five intra-assays. Day-to-day variation was evaluated by analyzing the standard methanol solution containing 1 nmol 4HB and urine containing 0.01 mg creatinine from a healthy child 10 times each over 8 weeks.

Recoveries were calculated by analyzing urine samples from a healthy child with or without addition of 0.2 nmol 4HB each five times.

The sensitivity limit of detection was defined as +3.0 SD above the measured blank average (n=5).

2.4. Urine samples

A urine sample from a child with 4-hydroxy-

butyric aciduria [2,3] and urine samples from 10 healthy disease-free subjects were analyzed.

3. Results

The coefficient of linear correlation (r^2) for calibration points between 0.05 and 1 nmol using standard solutions was 0.99, $y=0.016x-0.025$ (y, nmol; x, %). The coefficient of variation (C.V.) values were 7.4% for five intra-assays of 1 nmol methanol solution of 4HB and 5.7% for urine samples from a healthy control. The C.V. values in day-to-day variation over 8 weeks were 15.1% for 1 nmol 4HB and 6.8% for 4HB in a urine sample from a healthy control. Analytical recovery was 92.7% and the sensitivity limit was 0.03 nmol.

Urine 4HB concentration of a sample from a child with 4-hydroxybutyric aciduria was 1258 mmol/mol Cr (control, 0.28–2.97, mean 1.42 mmol/mol Cr) (Fig. 1).

4. Discussion

In organic acidemia screening, GC–MS analysis with conventional solvent extraction after acidification of urine samples may often miss the peak of 4HB due to lactonization. In a quality assessment study of urinary organic acid analysis, only nine of 18 laboratories detected 4-hydroxybutyric aciduria [13]. To prevent this ‘‘false negative result’’, we developed a sensitive method for urine 4HB measurement, which had no extraction procedure, but samples were only alkalinized with 10 μl of 0.1 N NaOH. Urease was also used because urea interfered with the peak of 4HB under our GC–MS conditions. Addition of NaOH was favorable not only for preventing conversion from 4HB to butylolactone,

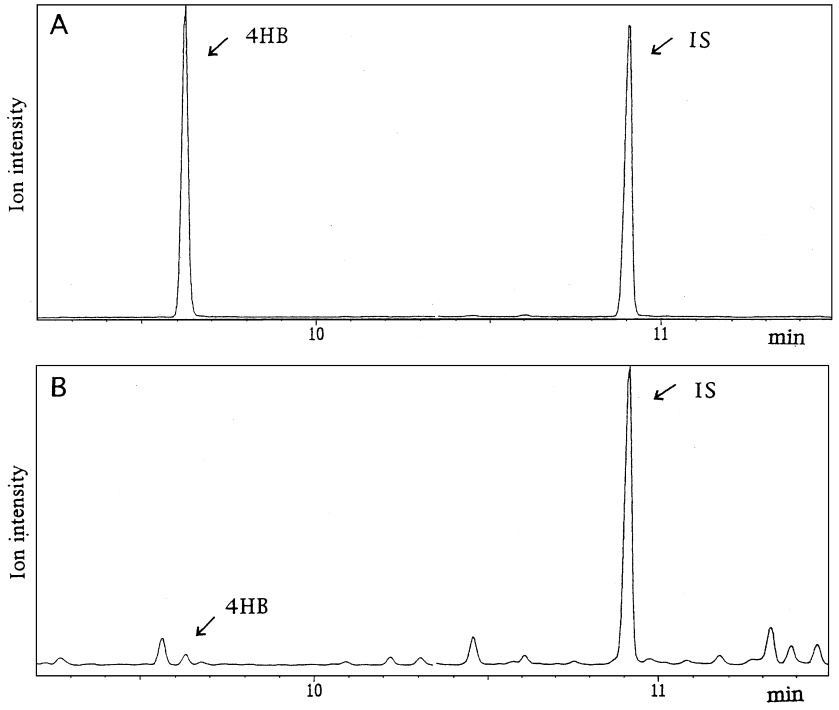

Fig. 1. Chromatograms of urine samples from a patient with 4-hydroxybutyric aciduria (A) and a normal control (B). Sample urine volume was 0.0002 mg creatinine equivalent in A and 0.01 mg creatinine equivalent in B. I.S., internal standard, 1 nmol dimethylsuccinic acid. In these particular chromatograms, the 4HB peak consisted of the intensity of m/z 233 ion in A and a summation of the intensity of m/z 233 and m/z 117 in B. The I.S. peak consisted of a summation of the intensity of m/z 231 and m/z 117 in both A and B.

but also preventing loss of 4HB and dimethylsuccinic acid during evaporation to form non-volatile salts [14].

Numerous methods for 4HB analysis have been developed mostly as a toxicological screening. Among them, simple methods, such as acetonitrile precipitation followed by TMS derivatization by Louagie et al. [9] and a liquid–liquid extraction using ethyl acetate followed by TMS derivatization [10,11], were reported. Recently, Shinka et al. reported 4HB measurement by GC–MS in which TMS derivatization after urease digestion of urine and deproteinization by ethanol [15]. Their sensitivity limit was 1 nmol/ml, 0.1 nmol for 100 μl of the sample amount in their method. In our method, after urease digestion, we alkalinized and then simply evaporated samples followed by derivatization. Our sensitivity limit was 0.03 nmol for the urine sample amount containing 0.01 mg creatinine, for example, 50 μl of urine at 20 mg/dl of creatinine. This simple and sensitive method can be useful for the diagnosis of 4-hydroxybutyric aciduria.

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