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Rapid and sensitive screening for and chemical diagnosis of Canavan disease by gas chromatography–mass spectrometry

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Abstract

The use of a rapid and sensitive assay for *N*-acetylaspartate (NAA) in urine or eluates from dried urine on filter paper to make a chemical diagnosis of Canavan disease (CD) is described. It involves a simplified urease pretreatment for sample preparation and gas chromatography–mass spectrometry (EI, scanning mode) with or without stable isotope dilution. Significant improvements in the recovery of NAA and the GC–MS data-handling device made the assay without stable isotope dilution sensitive and quantitative enough to diagnose CD: Its coefficient of variation (CV) was below 12%. The CV obtained with stable isotope dilution was below 9%. One patient with CD had an abnormal NAA level that was more than 6 S.D. above the mean of the age-matched controls. This diagnostic procedure is accurate for screening and for the chemical diagnosis of CD, with a good cost:benefit ratio. The urinary NAA levels of the healthy controls decreased significantly with age. This change should be considered in making a chemical diagnosis of this disease.

Keywords: Canavan disease; *N*-Acetylaspartate

1. Introduction

Canavan disease (CD, *N*-acetylaspartic aciduria; McKusick 271900) is an autosomal recessive inborn error of metabolism (IEM) caused by an aspartoacylase deficiency (EC 3.5.1.15). CD is a severe neurodegenerative disorder for which there is no effective treatment at present [1], although gene therapy is being investigated [2]. An increase of *N*-acetylaspartate (NAA) in urine was first described by Kvittingen and coworkers [3]. The quantitation of NAA in the urine from those suspected as having CD by solvent extraction and gas chromatography–mass spectrometry (GC–MS) with stable isotope dilution has been used for chemical diagnosis of the disease [4]. However, these techniques are suitable only for the measurement of limited metabolites, and not for the simultaneous measurement of different categories of metabolites in urine.

Here we describe a rapid, sensitive, and cost-effective method for mass screening and the chemical diagnosis of CD that involves urease pretreatment [5] and GC–MS, with and without stable isotope dilution. This method is applicable to the simultaneous measurement of various categories of metabolites.

2. Materials and methods

2.1. Subjects

Urine specimens that were collected from newborns for a pilot study of the newborn mass screening in Japan [6,7] were used as a newborn control group. Samples from another age-matched control group were from children who had been screened for the chemical diagnosis of IEM, but were found to be normal. The data for statistical analysis were divided into five groups according to the age of the controls: A (0 to 10 days, $n = 90$), B (11 days to 1 month, $n = 34$), C (1 month to 1 year, $n = 41$), D (1–4 years, $n = 76$), and E (4–12 years, $n = 42$).

Samples for the experimental group were taken from a Japanese female patient in whom CD had been diagnosed [8], from the members of her family, and from unrelated patients who had been suspected of having CD, but which was ruled out by chemical diagnosis.

Dried urine on filter paper was extracted with distilled water and used for the sample pretreatment procedure. Other

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urine samples were received frozen in dry ice and were stored at −20 °C prior to analysis.

2.2. Chemicals

d_3-*N*-Acetylaspartate (d_3-NAA) was from the Department of Chemistry, Columbia University (New York, NY). NAA and urease type III were from Sigma (St. Louis, MO). Other reagents were from Wako Pure Chemical Industry (Tokyo, Japan).

2.3. Preparation

2.3.1. Urease pretreatment method

The method of Matsumoto and Kuhara [5] and Kuhara [9] for the most advanced metabolic profiling was followed. To 100 μl of urine or extract from dried urine filter paper, 30 units of urease was added, and the mixture was incubated at 37 °C for 10 min. To this reaction mixture, 2-hydroxyundecanoate (2HC11), which we use for the internal standard for a number of metabolites in urine [9], was added as an internal standard to a final concentration of 250 nmol/ml. For the stable isotope dilution, 200 nmol/ml d_3-NAA was added to specimens as an extra internal standard. Samples were prepared as previously described [5]. Samples were converted to their trimethylsilyl derivatives (TMS) prior to GC–MS analysis.

2.3.2. Reference method: organic solvent extraction

As a reference method, the urinary NAA was extracted with diethyl ether and converted to TMS according to a previously reported method [10], with minor changes. Urine samples equivalent to 1 μmol creatinine, were used for the organic solvent extraction.

2.4. GC–MS

GC–MS analysis with the electron impact ionization and scanning from *m*/*z* 50 to 650 was carried out as previously described [5]. NAA was quantified from the data acquired.

2.5. Quantitation

Two peaks representing the NAA di- and tri-TMS were always detectable. Their mass spectra are shown in Fig. 1. The ratios of the two peak areas to that of the internal standard were examined by taking repeated measurements for 32 h using urine specimens spiked with the three different concentrations of NAA. Two methods for the quantitation of NAA were compared using samples spiked with 1.1–114 nmol of non-labeled NAA in 0.1 ml of control urine. The first method (non-stable isotope dilution method) quantified the sum of the total abundance obtained from the peak area of the ion at *m*/*z* 158 in the peak of NAA di-TMS and that at *m*/*z* 274 in the peak of NAA tri-TMS. In this case, the total abundance

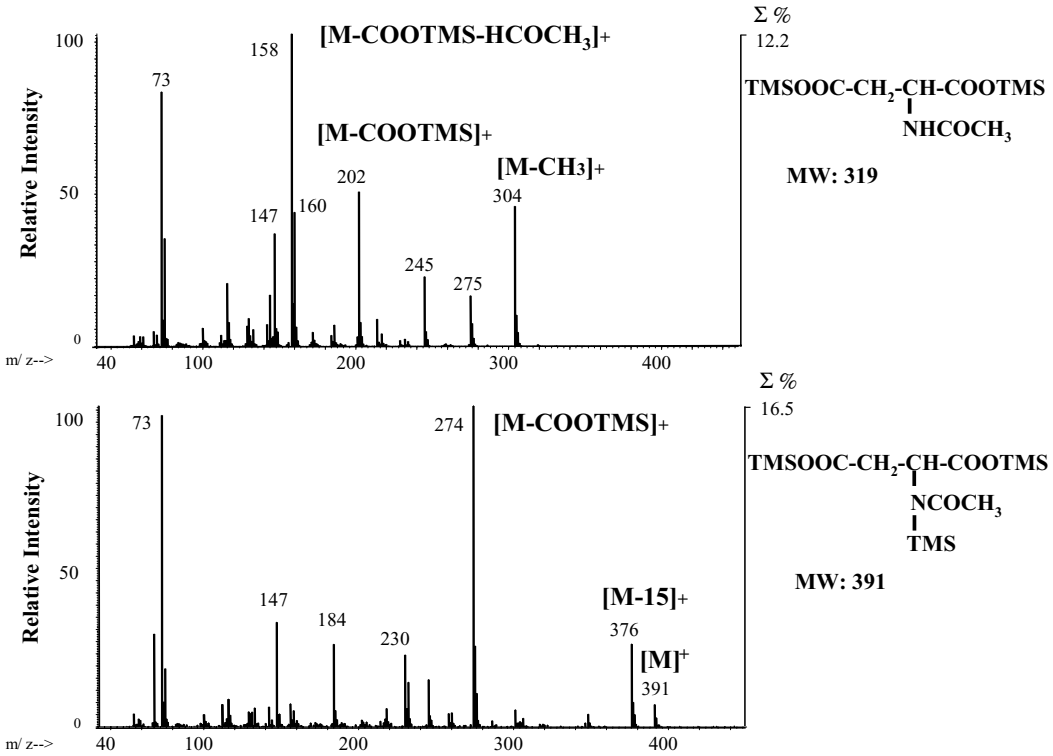

Fig. 1. Mass spectra of *N*-acetylaspartate di- and tri-TMS derivatives. The percentage of each ion's abundance to the total ion abundance (Σ%) is indicated on the vertical scale.

of the target ion for each peak was corrected by calculating the percentage of the total ion abundance of the spectrum (Σ%) (Fig. 1). The peaks of the fragment ion pair of *m*/*z* 158 for the di-TMS peak, *m*/*z* 274 for the tri-TMS peak, and *m*/*z* 229 for the internal standard peak were selected for calculating the concentration of NAA in urine. The second method (stable isotope dilution method) used the values calculated by the stable isotope dilution.

To determine the recovery of NAA and to validate the linearity and the inter-assay precision, the control urine was spiked with NAA of four different concentrations, from 11.4 to 1140 nmol/ml, and the specimens were analyzed.

Urinary creatinine was determined by the enzymatic method using a Synchron CX5 auto-analyser (Beckman Inst. La Brea, CA).

3. Results

3.1. Comparison of peak abundance of NAA obtained with two sample preparation methods

We examined whether the urease pretreatment method was superior to the organic solvent extraction method for the recovery of NAA, and hence the sensitivity of the NAA assay. Ten control urine specimens were prepared according to each method and measured by GC–MS with scanning mode under the same conditions. In all the samples prepared by the urease pretreatment method, NAA was determined quantitatively from 100 μl of urine, and the range and average creatinine content were 0.03–1.5 and 0.38 μmol creatinine. The sample volumes for the organic solvent extraction method varied from 0.14 to 1.50 ml, which were equivalent to 0.5–2.0 μmol creatinine. NAA could not be detected in four samples prepared by the latter method, even in a sample that was greater than 1 ml. In most of the urine samples, the NAA peak abundance using the urease pretreatment method was 10 times more intense than that using the organic solvent extraction method (Fig. 2).

3.2. Quantitation method

The ratio of the peak area of the di-TMS and tri-TMS to that of the internal standard was examined by repeating the measurements six times over 32 h using urine containing different concentrations of NAA (Fig. 3). The ratios were not always constant, but the sum of the ratio of each peak, corrected by the Σ%, was constant.

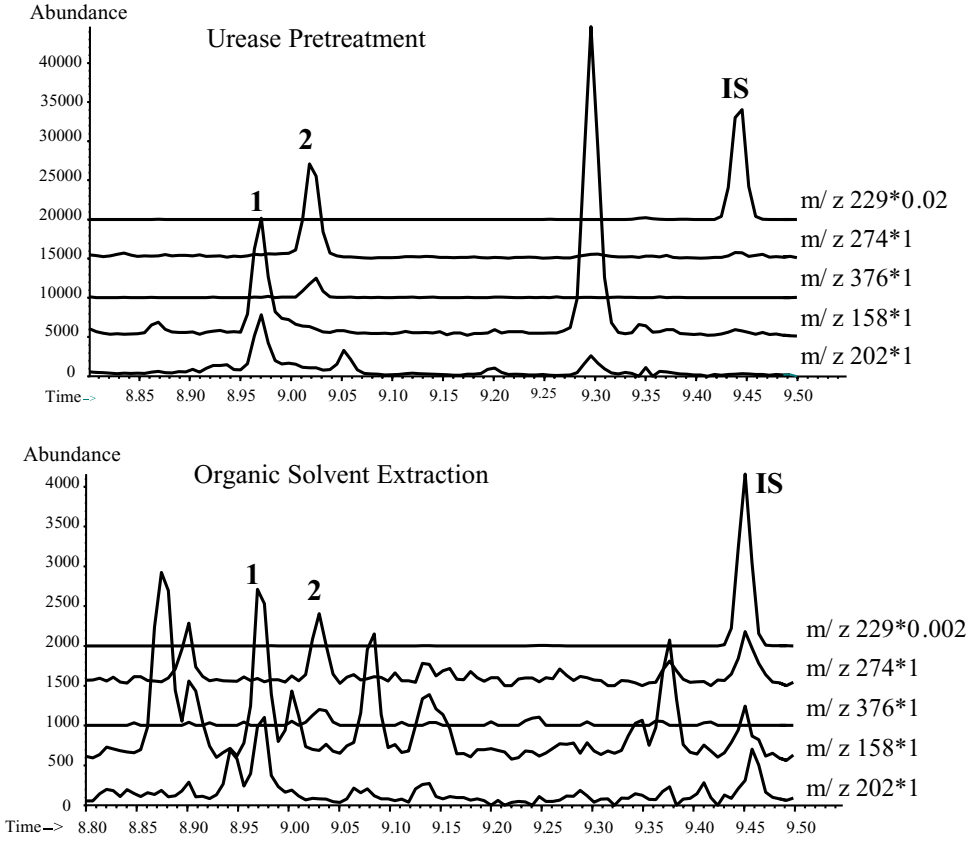

Fig. 2. Mass chromatograms of the target ions of the *N*-acetylaspartate TMS derivatives: urease pretreatment (upper panel) and the organic solvent extraction (lower panel). Urine specimens from the same healthy control were used for both. 25 nmol and 20 nmol of 2HC11 were added to 100 and 270 μl of urine in the upper and lower panels, respectively. Peaks 1, 2: *N*-acetylaspartate di- and tri-TMS. The target ions of *m*/*z* 158 and 202 at 8.98 min and the target ions of *m*/*z* 274 and 376 at 9.03 min were monitored for di- and tri-TMS, respectively. IS: 2HC11 was used as an internal standard; the target ion of *m*/*z* 229 at 9.45 min was monitored.

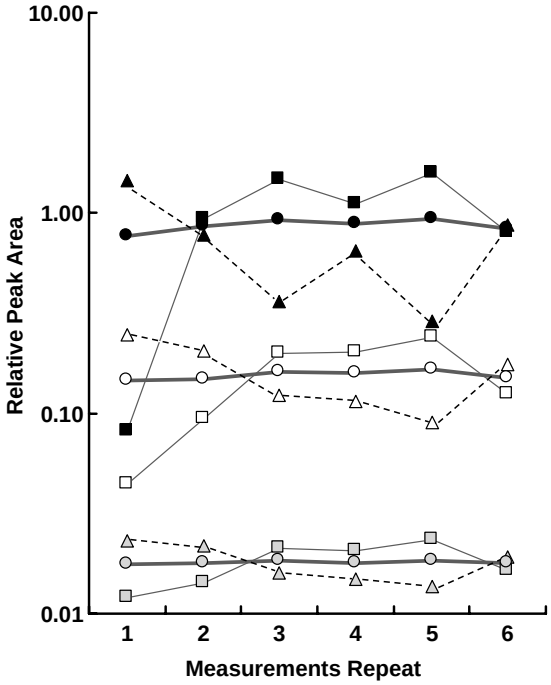

Fig. 3. Changes of the relative peak area of the di- and tri-TMS *N*-acetylaspartate compared with an internal standard in repeated measurements. Three different amounts of *N*-acetylaspartate were added to control urine specimens. (▲, ■, ●) 1140 nmol/ml; (△, □, ○) 228 nmol/ml; (△,□,□) 23 nmol/ml. None of the peak areas relative to that of the internal standard was constant, but the sum of the area of the 2 peaks, corrected by Σ%, relative to that of the internal standard was constant. (△) ratio of *m*/*z* 158/231 of internal standard; (□) ratio of *m*/*z* 274/231 of internal standard; (○) sum of peak of the 2 peak, after corrected by Σ%.

The monitored ions at *m*/*z* 158 in NAA di-TMS and at *m*/*z* 274 in NAA tri-TMS were used for quantitation without stable isotope dilution, after correction by Σ%. The corresponding fragment ions of the d_3-NAA TMS were *m*/*z* 161 and 277, respectively. However, the *m*/*z* 158 and 161 pair was influenced greatly by an isotope peak of the fragment ion of *m*/*z* 160, which was almost half as abundant as *m*/*z* 158 (Fig. 1). The ion pair of *m*/*z* 274 and 277 was used for quantitation with stable isotope dilution.

3.3. Accuracy and precision of the methods

The qualitative value of the NAA, calculated from the non-stable isotope dilution method, was linear up to 1140 nmol/ml. The equations for the regression line for the NAA found in urine (y) versus the NAA added to urine (x) was $y = 0.9617x - 22.72$; the correlation coefficient was 0.9894. The equation for the regression lines for the NAA calculated from the fragment ions of *m*/*z* 158 and 274 by the stable isotope dilution method were $y = 0.8519x + 16.1$ for *m*/*z* 158 and $y = 0.9101x + 6.0$ for *m*/*z* 274; the correlation coefficients were 0.9981 and 0.9996, respectively. The linearity calculated from the summation method without stable isotope dilution appeared equivalent to that obtained with stable isotope dilution.

The Spearman's correlation coefficient by rank (rs) of the NAA concentrations from 23 specimens calculated by both quantitative methods was 0.916. The mutual relationship between the two methods was sufficiently estimated from the results of the measurements.

The inter-assay precision of the non-stable isotope dilution method was indicated by coefficients of variation (CV) of 11.2, 6.0, 8.2, and 10.1% ($n = 5$–6) at a concentration of 11.4, 45.6, 228, and 1140 nmol/ml NAA, respectively, while that of the stable isotope dilution method for *m*/*z* 274 was shown by CVs of 6.3, 8.6, 5.6, and 2.0% ($n = 5$–6) (Table 1).

The extraction recovery of the NAA was determined by adding NAA at four known concentrations, 11.4, 45.6, 228, and 1140 nmol/ml, to control urine and comparing the difference to control urine that had not been spiked with NAA. The extraction recoveries obtained in these experiments were 72.6, 71.9, 75.2, and 93.0%, respectively.

3.4. NAA concentration in control urine

The concentration of NAA in 241 samples of control urine was calculated by the summation method. The data for statistical analysis were divided into five groups according to the age of the controls. Before proceeding with the statistical analysis, the residuals in the ANOVA were examined for a normal distribution by studying a histogram and a normal plot. After $\log_{10}$-transformation, the apparent residuals were found to be normally distributed, and thus for these measurements, $\log_{10}$-transformed data were used in the subsequent statistical analyses. A one-way ANOVA, Fisher's PLSD, and Scheffe's *F*-test were used for the statistical evaluation of differences between groups, with $P < 0.05$ considered significant. The urinary NAA levels of these groups showed statistically significant decreases with age, except

Table 1
Comparison of the inter-assay precision of two different quantification methods

Spiked NAA[a] (nmol/ml)	Sample preparations (*n*)	Mean of found NAA (nmol/ml)	CV (%)	Average CV (%)
Non-stable isotope dilution[b]				8.8
11.4	5	23.3	11.2	
45.6	6	47.8	6.0	
228	6	186	8.2	
1140	6	1074	10.1	
Stable isotope dilution[c]				5.6
11.4	5	27.5	6.3	
45.6	6	57.8	8.6	
228	6	239	5.6	
1140	6	1167	2.0	

[a] A control urine specimen was spiked with non-labeled NAA.

[b] Samples were measured under ordinary scanning mode and NAA levels were calculated by the sum of the total ion abundance obtained from the peak area of the ion at *m*/*z* 158 in the peak of NAA di-TMS and that of the ion at *m*/*z* 274 in the peak of NAA tri-TMS (see text).

[c] Samples were measured under ordinary scanning mode and NAA levels were calculated by stable isotope dilution from the peak area of the ions at *m*/*z* 274 and 277 in the peak of d_0- and d_3-NAA tri-TMS.

Table 2
Measurements of *N*-acetylaspartate in urine from patients and controls

Pretreatment method	Controls age[a] means ± S.D.[b] (*n*) (range)	Patients means ± S.D.[b] (*n*) (range)	Method				Reference (year)
			Sample volume (ml)	Stable isotope dilution	Target peak and derivatization	GC–MS mode	
Urease pretreatment	(A) 0D-10D 119 ± 56.8 (90) (42 − 364)		0.02–0.2	N	Di- and tri-TMS	Scanning	Present procedure
	(B) 11D-1M 92.1 ± 98.6 (34) (19.3 − 592)						
	(C) 1M-1Y 73.4 ± 46.1(41) (24.0 − 280)	1280, 1300 (1)					
	(D) 1Y-4Y 38.8 ± 19.2 (76) (13.4 − 93.3)	1380 (1)					
	(E) 4Y-12Y 19.4 ± 8.95 (42) (6.8 − 44.2)	1350 (1)					
Urease pretreatment		850–1260 (1)	0.02–0.2	Y	Di-or tri-TMS	Scanning	Present procedure
Solvent extraction	23.5 ± 16.1 (54) (4.1 − 59.2)	1441 ± 873 (95) (135–3686)	1	Y	[c] TMS	SIM	12 (1993)
Solvent extraction	3.57 ± 0.89 (4) (2.3 − 4.5)		[c]	Y	PFB	NCI SIM	15 (1992)
Solvent extraction	12.7 ± 7.8 (80) (6.6 − 35.4)	391–3073 (7)	1.8[d]	Y	Tri-TMS	SIM	14 (1991)
Solvent extraction	19.5 ± 10.3 (25) (6.6 − 35.4)	1491 (1)	1	Y	Di-TMS	NCI SIM	4 (1991)
Lyophilization	<30		0.1[d]	Y	Di-TMS	SIM	13 (1991)

TMS: trimethylsilated derivatives; PFB: pentafluorobenzyl ester derivatives; SIM: selective ion monitoring; NCI: negative chemical ionization.
[a] There was not a correct mention about age of controls in all previous reports.
[b] nmol/mmol creatinine.
[c] No description.
[d] A volume of urine containing Xμmole creatinine.

between the B and C groups. Non $\log_{10}$-transformed data are shown in Table 2. There was no statistically significant sex difference in the urinary NAA levels for any age group or for the total group.

3.5. NAA levels in the urine of a female patient, her family, and suspected patients

The NAA levels were determined in four urine specimens from a female Japanese patient, four from members of her family, and 12 from unrelated patients who had been suspected as having CD but ruled out by chemical diagnosis. The levels of urinary NAA are shown in Table 3, and were compared with the $\log_{10}$-transformed values of age-matched control groups. The abnormality expressed as the number *n* was obtained as described previously [11]. The patient with CD showed an abnormal NAA level that was more than 6 S.D. above the mean of the age-matched controls. The NAA levels from the members of the patient's family and the unrelated subjects with suspected CD, but which was ruled out by chemical diagnosis, did not show abnormal levels compared with the levels of the age-matched controls.

4. Discussion

Table 2 shows the NAA levels in urine from healthy controls and patients with CD from the present study and previous reports [4,12–15]. To improve the sensitivity, techniques, such as selected ion monitoring (SIM) [4,12–14], negative chemical ionization (NCI) [4,15], and/or pentafluorobenzyl ester (PFB) derivatization [15] have been used in these studies. However, these techniques are suitable only for the measurement of limited metabolites and not for the simultaneous measuring of different categories of metabolites in urine. The organic solvent extraction method has been used for many years to measure urinary NAA [4,10,14]. Another

Table 3
Urinary NAA levels in a female patient with Canavan disease, members of her family, and patients suspected of having CD

Group	Age	Concentration[a]	Abnormality (n)[b]
Patient	9M	1280 (1140)	6.8
($n = 1$)	1Y	1300 (1260)	8.5
	3Y9M	1380 (1140)	8.6
	4Y5M	1350 (850)	11.5
Father	39Y	8.5 (9.2)	−0.4
Mother	35Y	18 (19)	1.3
Sister	14Y	16 (12)	0.9
Sister	11Y	7.9 (8.0)	1.0
Suspected	<1Y ($n = 4$)	14–61 (14–34)	−2.0–0.9
Patients	<4Y ($n = 3$)	14–25 (14–27)	−0.9–0.4
	≥4Y ($n = 5$)	8–22 (7–20)	−0.2–1.7

[a] The values were obtained by two quantitation methods and expressed as nmol/mmol creatinine (see Section 2). The values in parentheses were obtained by the stable isotope dilution method.

[b] The measure of abnormality expressed as n was obtained as previously described [11]. $n = (X - \text{Mean})/SD$; X, $\log_{10}$-transformed concentration of NAA; Mean, $\log_{10}$-transformed mean in age-matched control; SD, $\log_{10}$-transformed SD in age-matched control.

procedure, urease pretreatment, was used in our neonatal IEM screening project [5]. With the latter procedure, it was possible to reduce the volume of specimens, the number of procedural steps, and the time required for sample preparation, and to increase the recovery of NAA, which contains a carboxyl and an acetamide group. A good recovery and increased sensitivity for NAA were obtained using urease pretreatment, as expected.

The ratio of the two peaks corresponding to di- and tri-TMS differed significantly between samples, and even for each injection of the same sample preparation. Under the stable isotope dilution condition, non-labeled and labeled NAA behaved in a similar manner, which enabled us to quantify it using one of the two TMS derivatives. When only one of these peaks was considered for the quantitation, quantitative data were not obtained with the non-stable isotope dilution method. As described previously, we devised a method for obtaining reproducible data by using 2HC11 as an internal standard that is added and used for the measurement of the number of metabolites present in urine [9]. The precision of the present method without stable isotope dilution seemed as good as that of the stable isotope dilution. Thus, we believe the urease pretreatment method using ordinary GC–MS measurement conditions is sensitive and quantitative enough to diagnose CD. We can investigate the incidence of CD by applying this procedure to the neonatal mass-screening project in Japan, without special modifications for the NAA assay.

Using organic solvent extraction, stable isotope dilution, and GC–MS with the SIM mode, NAA can be determined not only in urine, but also in amniotic fluid, CSF, and blood, and the measurement of NAA in amniotic fluid has been used for the prenatal diagnosis of CD [4]. The present method without stable isotope dilution is probably not suitable for measuring NAA in amniotic fluid, CSF, or blood, because the levels of NAA in these biological fluids are several times lower than in urine [4]. The precision was, however, sufficient for the assay of the urinary neonatal IEM screening project, and for accurate chemical diagnosis.

The levels of NAA in healthy controls <4 years old were higher than levels reported in earlier studies [4,12–15]. As the age of the controls was not clearly described in those reports, we cannot compare these results directly. Our results suggest that age-matched control data are necessary to evaluate the urinary NAA concentration in children <4 years old. The present study showed a statistically significant decrease with age in the urinary NAA levels of healthy controls. Thus, the use of age-matched controls is critically important for making an accurate chemical diagnosis of CD.

CD is found at high frequency in the Ashkenazi Jewish [12] and Arabic [13] populations, and a DNA-based diagnosis has recently been used to detect carriers in the former [16]. However, a few cases also occur in other ethnic groups [8,17], and different mutations are expected to give rise to the disease in different populations. To clarify the incidence of CD, it was necessary to develop a simple screening method that could be widely applied to a large number of people. Although there is no effective treatment for this disease at present, gene therapy is currently being investigated [2]. Should a gene therapy become available in the near future, the mass screening and rapid and accurate chemical diagnosis for CD will be indispensable for obtaining a diagnosis before onset.

Urine is superior to blood for diagnosis, not only because it is noninvasive, but also because there is enough NAA in urine to diagnose CD. The present study shows that urease pretreatment and GC–MS without stable isotope dilution but with a change in the data handling can be used to screen and make a chemical diagnosis of CD.

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