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Stable-isotope dilution gas chromatography–mass spectrometric measurement of 3-hydroxyglutaric acid, glutaric acid and related metabolites in body fluids of patients with glutaric aciduria type 1 found in newborn screening

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Abstract

We developed a simple and sensitive stable-isotope dilution method for the quantification of 3-hydroxyglutaric acid (3HGA) and glutaric acid (GA) in body fluids. In our method, *tert*-butyldimethylsilyl (tBDMS) derivatives of 3HGA and GA were measured with a conventional electron-impact ionization (EI) mode in gas chromatography–mass spectrometry (GC–MS). The control values for 3HGA in nmol/ml were 0.15 ± 0.08 (serum; $n = 10$) and 0.07 ± 0.03 (CSF; $n = 10$). In addition, glutarylcarnitine and free carnitine were quantified by electrospray tandem mass spectrometry. Using these methods, we monitored 3HGA, GA, and glutarylcarnitine in the body fluids of three patients with glutaric aciduria type 1 found during newborn screening. None of the patients had experienced neurological strokes, which are possibly caused by the accumulation of 3HGA, at 15–24 months of age under a disease-specific treatment, including carnitine supplementation. Our data showed that 3HGA levels were relatively high in some serum samples with lower glutarylcarnitine and carnitine levels, suggesting that carnitine supplementation may play a role in preventing the accumulation of 3HGA in patients with this disease.
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1. Introduction

Glutaric aciduria type 1 (GA 1; McKusick 231670) is an autosomal recessive disorder caused by mutations in the glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7) gene. The deficiency of GCDH in the metabolism of lysine, hydroxylysine, and tryptophan leads to the accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3HGA), glutaconic acid [1], and glutarylcarnitine [2] in body fluids. The clinical picture of this disease features abrupt neurological deterioration with focal striatal necrosis in infancy, often after infectious illnesses [3]. 3HGA is thought to be a possible cause of this phenomenon [4]. The main treatments for GA 1 are the restriction of amino acids as precursors of glutaryl-CoA, carnitine supplementation, and avoidance of fasting [3]. Such disease-specific therapy has improved the outcome of asymptomatic GA 1 patients found in a recent newborn-screening program in which the high glutarylcarnitine levels in dried blood spots of the patients were detected by tandem mass

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spectrometry (MS–MS) [3,5]. Thus, it is important to monitor the levels of 3HGA and the related metabolites in body fluids to evaluate the efficacy of the therapy.

Schor et al. reported a stable-isotope dilution method using ammonia chemical ionization and negative-ion/selected-ion monitoring as a sensitive and accurate GC–MS measurement of 3HGA [6]. Although this method is highly sensitive to quantify 3HGA levels in control CSF or amniotic fluid, it may not be practical to use ammonia as a reagent gas in routine laboratory work because it is difficult to handle. Thus, we developed a practical but sensitive method using a conventional electron-impact ionization (EI) mode for the GC–MS measurement of 3HGA and GA as *tert*-butyldimethylsilyl (tBDMS) derivatives. In our method, the gas chromatographic separation between 3HGA and 2-hydroxyglutaric acid (2HGA), which is increased in 2-hydroxyglutaric acidurias [7], was achieved using a capillary column with a polar liquid phase. Using this method, we measured the related metabolites in the body fluids of three GA 1 patients who were identified in our MS–MS newborn-screening program.

2. Experimental

2.1. Materials

2.1.1. Biological samples

Three Japanese patients with GA 1 (MM, HK, and CH) were identified during MS–MS newborn screening. The glutarylcarnitine levels in dried blood spots of the patients were 2.22, 4.40, and 1.95 nmol/ml, respectively, with a cutoff of 0.8 nmol/ml. GCDH activities in lymphocytes [8] in all patients were below the detection limit of 0.10 pmol/min/10^6 cells, with a control range of 3.43–6.15. CSF samples were collected from all patients twice, before therapy and 3–12 months later. Informed consent was obtained before sample collection.

All the patients had been treated with protein-restricted diets, carnitine supplementation, and prompt intravenous fluid infusions in cases of decreased caloric intake due to febrile illnesses or gastroenteritis. Patient MM was floppy and had delayed development. Her developmental quotient (DQ) was 50 during her early infancy, but it had markedly improved to 96 by the time she was 24 months. Patients HK and CH showed normal development up to the age of 20 and 15 months, respectively.

Frozen serum, CSF, and urine specimens were transferred to our laboratory and stored in a freezer until analysis. Reference body fluid samples were obtained from hospitalized children, ages 2 months to 14 years, who did not have GA 1.

2.1.2. Chemicals

3-Hydroxyglutaric acid and 3-hydroxy[$^2H_4$]glutaric acid were purchased from the VU Medical Center Metabolic Laboratory (Amsterdam, The Netherlands). [$^2H_4$]Glutaric acid and [$^2H_3$]carnitine were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). [$^2H_9$]Glutarylcarnitine was synthesized in our laboratory [9]. Glutaric acid, 2-hydroxyglutaric acid, butanolic HCl (10%), HPLC-grade acetonitrile, methanol, and distilled water were purchased from Nacalai Tesque (Kyoto, Japan). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was purchased from GL Science (Tokyo, Japan).

2.2. Methods

2.2.1. Sample preparation

GA and 3HGA concentrations in body fluids were determined by isotope-dilution methods. A mixture (0.45 ml) containing 0.05–0.2 ml of serum or CSF, 0.2 ml of saturated NaCl solution, 0.01 ml of 4N HCl, 0.02 ml of 2 mg/L of [$^2H_4$]3HGA, and 0.02 ml of 4 mg/L of [$^2H_4$]GA was extracted twice with 2 ml of ethylacetate. The combined solvent layer was dried under an N_2 stream, and the dry residue was dissolved in 0.04 ml of MTBSTFA and heated at 100 °C for 30 min. For the analysis of urine samples, a mixture of an aliquot of urine containing 0.005–0.01 mg of creatinine (Cr), 0.02 ml of 0.05 mg/ml of [$^2H_4$]3HGA, and 0.02 ml of 0.1 mg/ml of [$^2H_4$]GA was incubated with urease at 37 °C for 15 min and deproteinized using methanol [10]. The supernatant was dried under an N_2 stream, and the dry residue was dissolved in 0.1 ml of MTBSTFA and heated at 100 °C for 30 min.

For recovery determination, a mixture containing 0.1 ml of control serum, spiked with 0.005, 0.01, or 0.02 ml of 2 mg/L of 3HGA and 4 mg/L of GA, 0.2 ml of saturated NaCl solution, and 0.01 ml of 4N HCl was extracted with ethylacetate. The extract combined with 0.02 ml of 2 mg/L of [$^2H_4$]3HGA and 0.02 ml of 4 mg/L of [$^2H_4$]GA was dried and derivatized. On the other hand, a mixture of 0.005, 0.01, or 0.02 ml of 2 mg/L of 3HGA and 4 mg/L of GA, 0.02 ml of 2 mg/L of [$^2H_4$]3HGA, and 0.02 ml of 4 mg/L of [$^2H_4$]GA was dried directly and derivatized. For the test with the respective amount of spiked 3HGA and GA, three samples of each were measured by GC–MS, and the mean values were compared. Correction with the amounts of 3HGA and GA in the control serum specimen was done.

For acylcarnitine measurements by MS–MS [11], a mixture of 0.012 ml of the sample and 0.44 ml of methanol solution with stable isotope-labeled acylcarnitines, including [$^2H_9$]glutarylcarnitine and [$^2H_3$] carnitine, was centrifuged. The supernatant was dried and derivatized with butanolic HCl, dried again, and then dissolved in 50% acetonitrile.

2.2.2. Gas chromatography and mass spectrometry conditions

For serum or CSF samples, gas chromatography–mass spectrometry (GC–MS) analysis was performed on a model QP5050 gas chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved on

a capillary column, SPB-50 (30 m × 0.25 mm inner diameter, film thickness 0.25 μm) (Supelco, Tokyo, Japan). One microliter of the processed samples was injected using the splitless mode at a temperature of 280 °C. The oven temperature was programmed to rise from 50 to 170 °C at a rate of 10 °C/min, to 230 °C at 5 °C/min, and to 290 °C at 10 °C/min. The mass spectrometer was operated under a positive EI mode, and the intensities of the ion, *m/z* 433 for 3HGA, *m/z* 437 for [$^2H_4$]3HGA, *m/z* 303 for GA, and *m/z* 307 for [$^2H_4$]GA, were recorded using a selected ion monitoring (SIM) mode. For urine samples, GC–MS analysis was performed on a model SSQ710 single-stage mass spectrometer (ThermoElectron, Yokohama, Japan) equipped with a model HP5890 gas chromatograph (Yokogawa, Tokyo, Japan). The measurement conditions were the same as those in the measurement by QP5050.

2.2.3. Tandem mass spectrometry conditions

Acylcarnitines were analyzed by electrospray MS–MS using a model TSQ7000 triple-stage mass spectrometer (ThermoElectron, Yokohama, Japan) [11]. Flow injection analysis was done. Precursor ion scanning using a product ion *m/z* 85 was performed, and the peak heights of *m/z* 218 for carnitine, *m/z* 221 for [$^2H_3$]carnitine, *m/z* 388 for glutarylcarnitine, and 397 for [$^2H_9$]glutarylcarnitine were used for quantification.

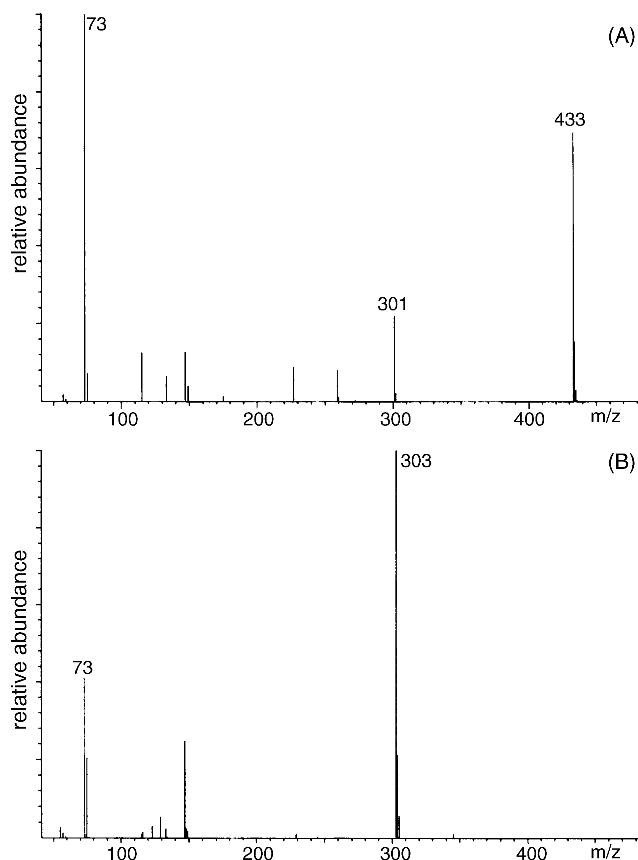

Fig. 1. Electron-impact ionization mass spectra of *tert*-butyldimethylsilyl derivatives of 3HGA (A) and GA (B).

3. Results

The mass spectra of 3HGA and GA as tBDMS derivatives under positive EI conditions are shown in Fig. 1. The fragment ions $[M-57]^+$, *m/z* 433 for 3HGA and *m/z* 303 for GA, were identified as prominent ions. The labeled 3HGA and GA showed the ions $[M-57]^+$, *m/z* 437 and 307, respectively (data not shown). Typical SIM chromatograms of a control serum sample are shown in Fig. 2. Quantification was performed using peak areas of the designated ion on SIM chromatograms. Using QP5050 GC–MS, the linearity of the calibration curve for added 3HGA was observed over the concentration range 0.017–3.4 nmol/ml: slope = 0.85, in-

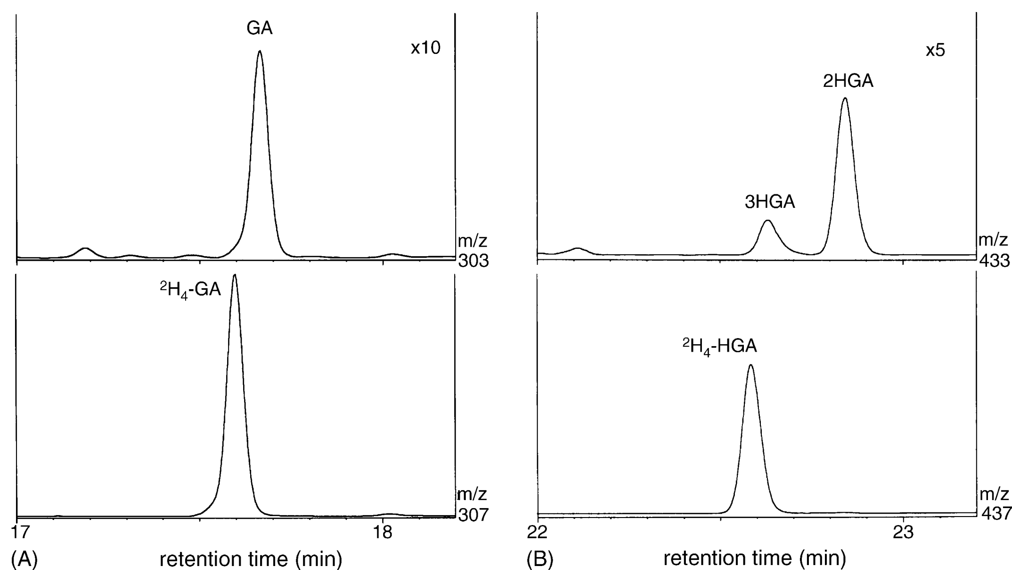

Fig. 2. Selected ion chromatograms of 3HGA and GA in a control serum sample. 2HGA, 2-hydroxyglutaric acid.

Table 1
Intra- and inter-assay variablities of 3HGA and GA determinations using a control serum specimen with and without spiked 3HGA and GA or a patient's urine specimen

	Serum (nmol/ml)		Urine (mmol/mol Cr)
Intra-assay ($n = 6$)			
3HGA (CV)	0.032 ± 0.0022 (6.9%)	1.42 ± 0.030 (2.1%)	21 ± 1.18 (5.6%)
GA (CV)	0.27 ± 0.0095 (3.5%)	3.4 ± 0.065 (1.9%)	47 ± 1.97 (4.2%)
Inter-assay ($n = 6$)			
3HGA (CV)	0.034 ± 0.0038 (11.2%)	1.38 ± 0.052 (3.8%)	22 ± 1.56 (7.1%)
GA (CV)	0.25 ± 0.013 (5.2%)	3.1 ± 0.130 (4.2%)	45 ± 3.02 (6.7%)

CV: coefficients of variation.

tercept = 0.008, and $r^2 = 0.999$ and, for added GA, over the concentration range 0.03–30 nmol/ml: slope = 0.97, intercept = 0.016, and $r^2 = 0.998$. Using SSQ710 GC–MS, the linearity of the calibration curve for added 3HGA was observed over the concentration range 0.34–270 nmol/ml: slope = 0.86, intercept = 0.77, and $r^2 = 0.999$ and for added GA, over the concentration range 1.5–760 nmol/ml: slope = 0.95, intercept = 1.6, and $r^2 = 0.998$.

The limits of quantification (signal-to-noise ratio of 10:1) for 3HGA and GA in aqueous solution were 0.017 and 0.0019 nmol/ml, respectively, using QP5050 GC–MS, and 0.34 and 0.057 nmol/ml, respectively, using SSQ710 GC–MS.

The recovery for 3HGA was 47, 48, or 51% at the concentration of 0.68, 1.35, or 2.7 nmol/ml, and that for GA was 85, 86, or 83% at 1.52, 3.0, or 6.1 nmol/ml, respectively.

The intra-assay variabilities were tested by the analysis of six samples processed at the same time using a control serum specimen with or without spiked 3HGA and GA or a patient's urine specimen, and the inter-assay variabilities were tested by processing and analyzing one sample on 6 different days. The coefficients of variation are listed in Table 1.

The control ($n = 10$) values for 3HGA in serum (nmol/ml), CSF (nmol/ml), and urine (mmol/mol Cr) were 0.15 ± 0.08, 0.07 ± 0.03, and 1.3 ± 0.7, respectively, and those for GA were 0.68 ± 0.36, 0.93 ± 0.38, and 3.8 ± 2.0, respectively. Those for glutarylcarnitine in serum (nmol/ml) and CSF (nmol/ml) were <0.3 and <0.1, respectively.

The concentrations of 3HGA and GA in urine, serum, and CSF samples of the three patients with GA 1 are shown in Fig. 3. The amounts of urinary GA of all three patients were large during the newborn period. Those of patient HK remained high, while those of the other patients decreased markedly during treatment. Regardless of the urinary GA levels of the patients, moderate amounts of urinary 3HGA were observed during infancy. A similar time course was observed in the patients' serum levels of 3HGA and GA. The serum levels of 3HGA in patient MM were similar to those in patient HK, although the serum levels of GA in patient HK were markedly higher than those in patient MM. The CSF levels of 3HGA in the patients were kept low during the newborn period and later infancy, although those of GA were markedly high.

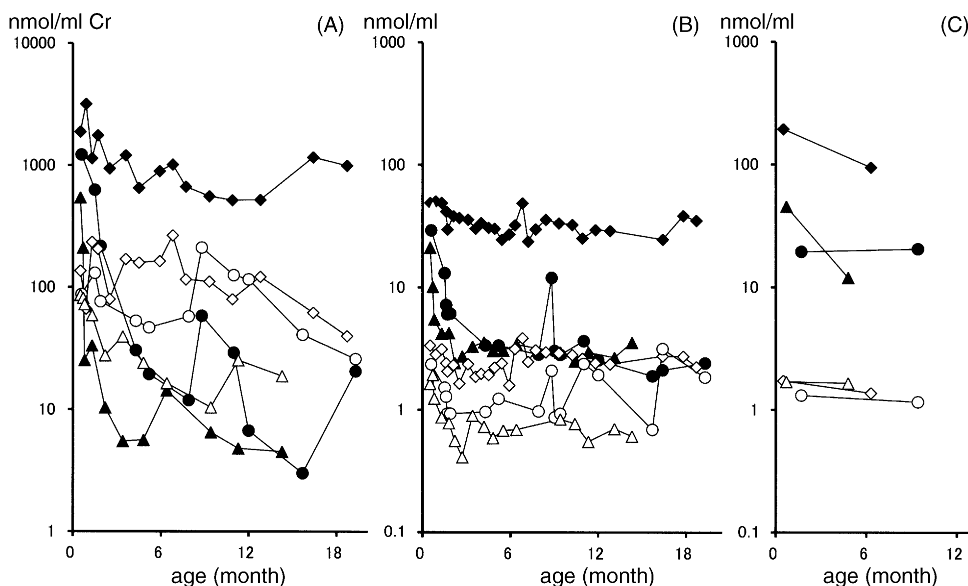

Fig. 3. 3HGA and GA levels in urine (A), plasma (B), and CSF (C) of three patients with GA 1. Open circle for 3HGA and closed circle for GA in patient MM, open diamond for 3HGA and closed diamond for GA in HK, and open triangle for 3HGA and closed triangle for GA in CH.

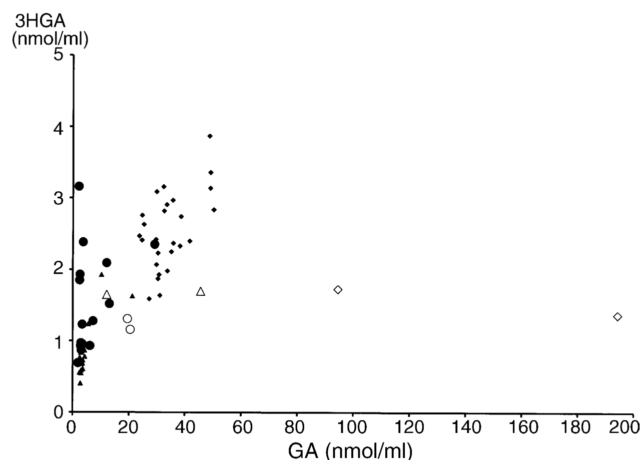

Fig. 4. The relationship between 3HGA and GA levels in serum and CSF. Closed circle for serum and open circle for CSF of patient MM, closed diamond for serum and open diamond for CSF of HK, and closed triangle for serum and open triangle for CSF of CH.

The relationship between the patients' 3HGA and GA levels in serum or CSF is shown in Fig. 4. Although the concentrations of 3HGA were roughly related to those of GA in most serum samples, those of 3HGA in some serum samples of patient MM were relatively high compared with those of GA. Furthermore, the 3HGA concentrations in the CSF of all patients were distributed at similar low levels regardless of GA levels.

The relationship between 3HGA and glutarylcarnitine or free carnitine concentrations in the serum or CSF of the patients is shown in Fig. 4. In most samples, the 3HGA concentrations were lower than the glutarylcarnitine concentrations. The 3HGA concentrations in some serum samples of patient MM, however, were higher than the glutarylcarnitine concentrations. Although 3HGA and free carnitine levels were not correlated, those of 3HGA were high when those of free carnitine were low in some serum samples of patients MM and CH (Fig. 5).

4. Discussion

In GA 1 patients, glutaryl-CoA is accumulated due to the deficiency of glutaryl-CoA dehydrogenase. The accumulation of glutaryl-CoA leads elevated levels of 3HGA, GA, and glutarylcarnitine in body fluids [1,2]. Although the exact mechanism of the formation of 3HGA is not known, 3HGA accumulation in the central nervous system is thought to be a possible cause of neurological deterioration with focal striatal necrosis in infancy [4]. Glutarylcarnitine is formed through the combination of glutaryl-CoA and carnitine, and the urinary excretion of glutarylcarnitine leads to carnitine deficiency [2]. Although the supplementation of carnitine is thought to be an effective therapy for GA 1 patients [3], the mechanism has not been clarified. Thus, 3HGA and the related metabolites should be monitored as indicators of metabolic derangement in GA 1 patients.

We have developed a new stable-isotope dilution method for the measurement of 3HGA and GA in body fluids as tBDMS derivatives. This method is sensitive enough for use in routine laboratory work, especially for the analysis in GA 1 patients. The limit of quantification of 0.017 nmol/ml of 3HGA in serum or CSF of this method, using 0.2 ml of specimen, is comparable with that of 0.01 nmol/ml in the reported method, the chemical ionization mode using ammonia as a

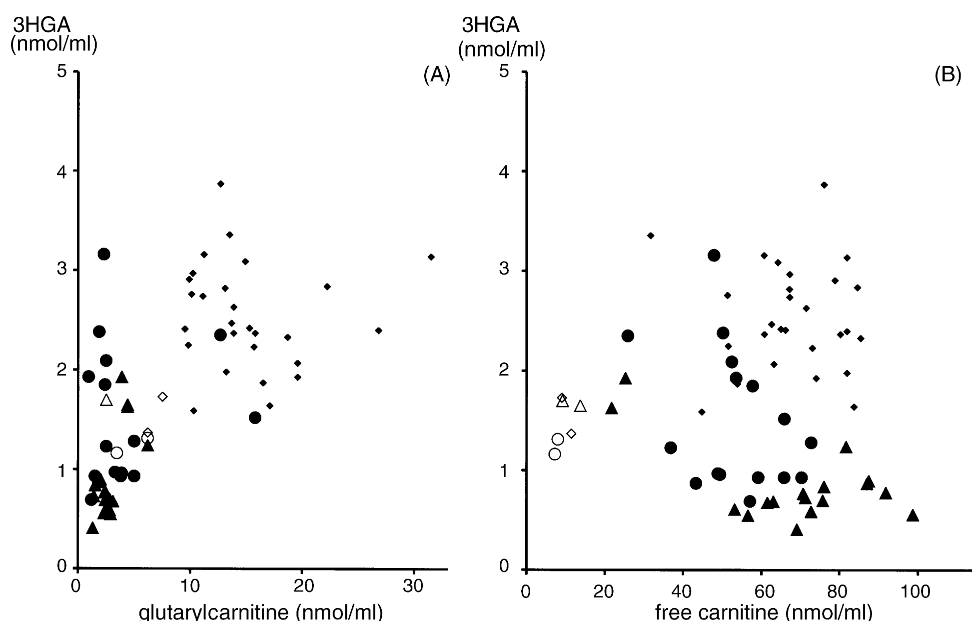

Fig. 5. The relationship between 3HGA and glutarylcarnitine levels (A) and between 3HGA and free carnitine levels (B) in serum and CSF. Closed circle for serum and open circle for CSF of patient MM, closed diamond for serum and open diamond for CSF of HK, and closed triangle for serum and open triangle for CSF of CH.

reagent gas and 1.0 ml of processed specimen [6]. Under a positive EI mode, 3HGA and GA as tBDMS derivatives produce positive fragment ions of $[M-57]^+$ with higher intensities in higher mass numbers than those produced by trimethylsily derivatives. We used an initial GC oven temperature as low as 50 °C to increase the sample introduction into the capillary column by splitless injection. In addition, in our method, baseline separation between 3HGA and 2-hydroxyglutaric acid as tBDMS derivatives was achieved using a capillary column with a polar liquid phase, as reported previously [6], for reliable and accurate quantification.

Our method is simple, since both the hydroxy and carboxy groups of 3HGA are derivatized using a single reagent. It is unnecessary to use ammonia as a reagent gas, which is difficult to regulate in the laboratory. Although we adopted a convenient solvent extraction method in which the recovery of 3HGA was about 50%, some increment in sensitivity can be obtained if solid-phase extraction is used [1]. Indeed, we derivatized urease-treated urine specimens without the extraction process and were able to perform the sensitive analysis using an aliquot of control urine containing levels as low as 0.01 mg Cr.

Patient HK showed large amounts of GA in urine and was thought to be a high excretor of GA [1], while the amounts of GA in the other patients decreased later in infancy. In GA 1 patients, 3HGA in urine is thought to be disease-specific and the only diagnostic metabolite [3]. The amounts of 3HGA in the urine of our patients, even later in infancy, were considerably higher than those of controls. The changes in the levels of 3HGA and GA in the patients' serum were similar to those in urine. However, striatal injury, which is characteristic of GA 1, can be associated with any excretion patterns of organic acids [3]. Thus, the measurement of the related metabolites in CSF or brain tissues seems important for evaluating the pathological effects on the central nervous system.

Recently, region-specific analysis of organic acids and acylcarnitines in the post-mortem brain of a GA 1 patient who reportedly had not been treated with carnitine revealed higher concentrations of 3HGA than those of GA or glutarylcarnitine in the putamen [4]. Indeed, the glutarylcarnitine levels in CSF of this patient were rather lower than those shown in our study, while the 3HGA levels were much higher than those in our patients. This finding may be due partly to a secondary carnitine deficiency of the patient and partly to the use of stable isotope-labeled octanoylcarnitine as an internal standard to quantify glutarylcarnitine. Since the intensity of product ion m/z 85 produced from glutarylcarnitine in the collision-induced dissociation process of MS–MS measurement is considerably lower than that from octanoylcarnitine, the glutarylcarnitine values in the report mentioned above are thought to be lower than those in our study using labeled glutarylcarnitine as an internal standard.

Carnitine is reportedly transported across the blood–brain barrier by a low-affinity carrier system [12,13], and it is synthesized and used locally in the brain [14]. We observed increased levels of carnitine in the CSF of a patient with propionic acidemia after carnitine supplementation [15]. In the present study, carnitine levels in CSF of the patients with GA 1 were not markedly increased by carnitine supplementation. Nevertheless, it is possible that carnitine supplementation at least prevented a secondary carnitine deficiency in these patients. In addition, it is worth mentioning that the 3HGA levels were relatively high in some serum samples that contained low levels of carnitine because they were collected when the patients did not take carnitine due to infectious diseases or other problems. Thus, our data suggest that carnitine supplementation may play an important role in preventing the accumulation of 3HGA not only in serum but also in CSF, that is, in the central nervous system.

Fortunately, our GA 1 patients identified during MS–MS newborn screening have not shown any neurological strokes. The present study did not show whether 3HGA levels in CSF are related to central nervous system abnormalities. Further careful monitoring of the levels of the related metabolites in CSF as well as in the other body fluids is needed.

In conclusion, our method for 3HGA and GA promotes an adequate evaluation of GA 1 patients identified in newborn screening.

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