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In vitro probe acylcarnitine profiling assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry predicts severity of patients with glutaric aciduria type 2☆

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ABSTRACT

Glutaric aciduria type 2 (multiple acyl-CoA dehydrogenase deficiency, MAD) is a multiple defect of mitochondrial acyl-CoA dehydrogenases due to a deficiency of electron transfer flavoprotein (ETF) or ETF dehydrogenase. The clinical spectrum are relatively wide from the neonatal onset, severe form (MAD-S) to the late-onset, milder form (MAD-M). In the present study, we determined whether the in vitro probe acylcarnitine assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry (MS/MS) can evaluate their clinical severity or not. Incubation of cells from MAD-S patients with palmitic acid showed large increase in palmitoylcarnitine (C16), whereas the downstream acylcarnitines; C14, C12, C10 or C8 as well as C2, were extremely low. In contrast, accumulation of C16 was smaller while the amount of downstream metabolites was higher in fibroblasts from MAD-M compared to MAD-S. The ratio of C16/C14, C16/C12, or C16/C10, in the culture medium was significantly higher in MAD-S compared with that in MAD-M. Loading octanoic acid or myristic acid led to a significant elevation in C8 or C12, respectively in MAD-S, while their effects were less pronounced in MAD-M. In conclusion, it is possible to distinguish MAD-S and MAD-M by in vitro probe acylcarnitine profiling assay with various fatty acids as substrates. This strategy may be applicable for other metabolic disorders.

1. Introduction

Fatty acid oxidation disorders (FAODs) potentially cause energy crises that are triggered by stress brought on by starvation or infection, and manifest themselves through nonketotic hypoglycemia, acute encephalopathy, or symptoms similar to those of Reye's syndrome. Among FAODs, medium-chain acyl-CoA dehydrogenase deficiency (MCAD-def) is most common among Caucasian [1], whereas very long-chain acyl-CoA dehydrogenase deficiency (VLCAD-def) and carnitine palmitoyl-CoA transferase 2 deficiency (CPT2-def) are common in Japanese, followed by glutaric aciduria type 2 (multiple acyl-CoA dehydrogenase deficiency: MAD) [2].

In MAD, multiple mitochondrial FAD-dependent dehydrogenases are impaired due to a defect in α- or β-subunits of electron transfer flavoprotein (ETF-α and ETF-β; OMIM 608053 and 130410 respectively) or ETF dehydrogenase (ETF-DH; OMIM 231675) [3,4]. The clinical forms of MAD include the neonatal-onset form (severe form: MAD-S) and the late-onset form (milder form: MAD-M). MAD-S occurs during the neonatal period, and is fatal. MAD-M often becomes symptomatic after infancy, and has episodic symptoms of hypotonia, tachypnea, skeletal muscle symptoms such as myalgia or rhabdomyolysis, and biochemical abnormalities including liver dysfunction, hypoglycemia, or hyperammonemia [5]. These 2 clinical forms can be sharply separated. Biochemical diagnosis is made by blood acylcarnitine analysis using electrospray ionization tandem mass spectrometry (MS/MS) that measures increases in C4 to C18 acylcarnitine, or through urinary organic acid analysis using gas chromatography mass spectrometry (GC/MS) that detects an increase of ethylmalonic acid, glutaric acid, isovalerylglycine, or dicarboxylic acids. However, it is not always feasible to make a definitive diagnosis of MAD by GC/MS or MS/MS, especially during the stable phase of MAD-M [6]. While genetic diagnosis may represent alternative strategy to make accurate diagnosis of MAD, the genotype/phenotype correlation is not clear.

The usefulness of in vitro probe acylcarnitine assay using cultured fibroblasts and MS/MS for the diagnosis of many FAODs has recently been reported [7]. Other reports showed that severity are associated with acylcarnitine prolife in deficiencies of MCAD, VLCAD, CPT2 and long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) [8–12]. Subsequent reports also demonstrated that it

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is feasible to distinguish deficiency with carnitine–acylcarnitine translocase (CACT) from CPT2 as well as LCHAD from MTP deficiency [13,14]. Herein, we investigated if the severity of MAD can be determined by in vitro probe acylcarnitine assay.

2. Materials and methods

2.1. Patients with MAD

The 14 patients with MAD were studied. MAD was biochemically diagnosed using GC/MS and/or MS/MS, and further conclusively diagnosed using gene analysis and immunoblotting. Their clinical features were previously described [15]. They included four cases with ETF-α deficiency, two subjects with ETF-β deficiency, and eight patients with ETF-DH deficiency. The clinical phenotype included 3 cases with MAD-S, 10 subjects with MAD-M, and 1 asymptomatic child who was detected before disease onset by the neonatal screening. No obvious correlation between clinical severity and the specific defective enzyme was seen. All 3 cases with MAD-S died during the neonatal period. Seven of the 10 cases of MAD-M developed the disease during infancy with nonketotic hypoglycemia, acute encephalopathy, or Reye-like syndrome. The remaining 3 cases with MAD-M showed muscle symptoms such as myalgia and rhabdomyolysis or occasional general fatigue in later childhood or later.

2.2. Cultured fibroblasts

Fibroblasts from 14 Japanese patients with MAD were used. We also analyzed cultured fibroblasts from 4 healthy controls, one case each of MCAD-def, VLCAD-def, CPT2-def, or mitochondrial trifunctional protein deficiency (MTP-def) and primary carnitine deficiency (PCD) to validate the specificity of our in vitro probe acylcarnitine assay using cultured fibroblasts with MS/MS and to compare with MAD samples. Cells were cultured in modified eagle medium (MEM; Nissui) with 2 mM L-glutamine, 10% BSA (Sigma) and 1% penicillin/streptomycin until achieving confluency at 37 °C and 5% of CO_2.

2.3. In vitro probe acylcarnitine assay of fibroblasts using MS/MS

An in vitro probe assay was performed as described by J.G. Okun et al. with some minor modification [8]. Briefly, the cultured fibroblasts were seeded into a 6-well plate, and washed twice with PBS when they reached confluent. Cells were subsequently cultured in MEM loaded with 0.2 mM palmitic acid. In some experiments, palmitic acid was replaced with either octanoic acid or myristic acid. After incubating for 96 h, the culture medium was collected to analyze acylcarnitines by MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Statistic analysis was performed using Mann–Whitney's *U*-test.

3. Results

3.1. Acylcarnitine in cultured fibroblasts with MAD-S and MAD-M shows distinct profile

In the pilot experiments, we confirmed that our in vitro acylcarnitine probe assay demonstrates specific metabolic profile for

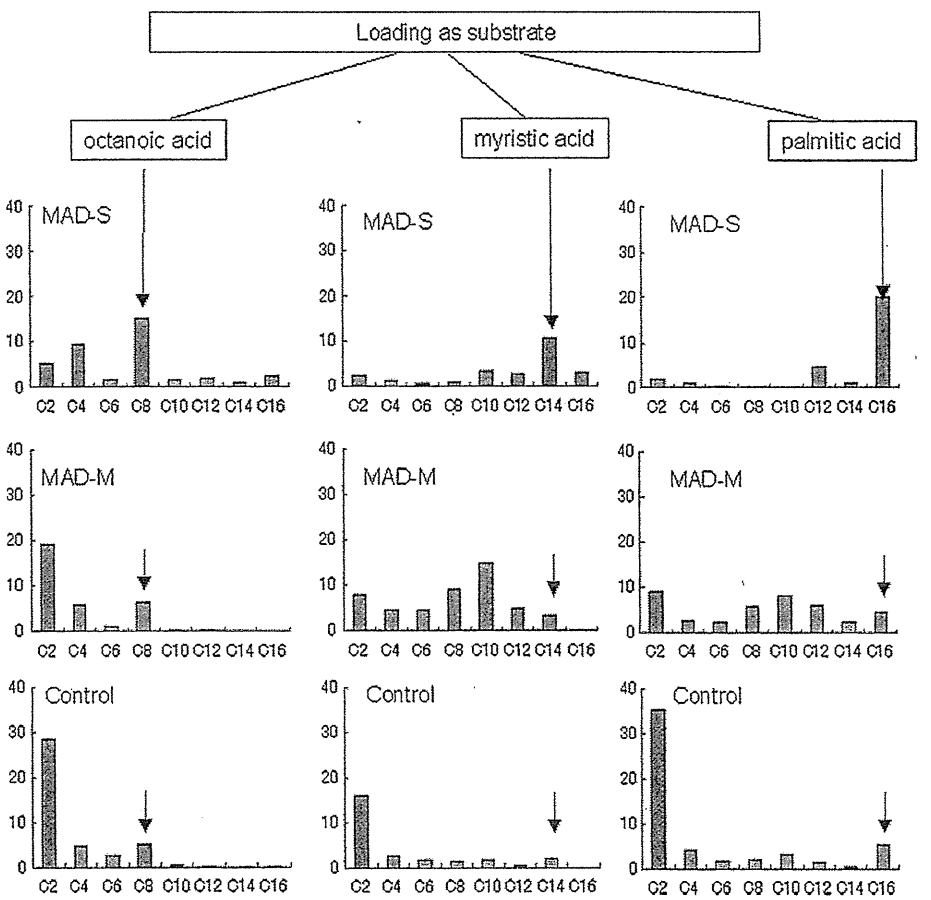

Fig. 1. The acylcarnitine profiles of fibroblasts from MAD-S and MAD-M. Arrows indicate loaded fatty acids; octanoic acid, myristic acid or palmitic acid. The *Y* axis represents values of acylcarnitines expressed as nmol/mg protein/96 h.

Table 1
Acylcarnitine profiles in culture medium of fibroblasts from MAD patients incubated with unlabelled palmitic acid for 96 h.

Clinical type	Acylcarnitine ratio; mean ± SEM				
	Palmitic acid				
	C16/C14	C16/C12	C16/C10	C16/C8	C16/C2
MAD-S (n = 3)	30.1 ± 16.9[*,†] (4.08–61.8)	6.02 ± 1.08[*,†] (3.85–7.12)	59.0 ± 24.6[*,†] (9.85–84.0)	69.1 ± 17.9[*,†] (33.5–89.5)	5.28 ± 1.75[*,†] (1.81–7.48)
MAD-M (n = 10)	4.33 ± 0.83 (1.52–10.0)	0.97 ± 0.17 (0.31–2.11)	1.05 ± 0.58 (0.22–6.20)	1.19 ± 0.70 (0.00–7.38)	0.73 ± 0.43[**] (0.08–4.57)
Asymptomatic (n = 1)	1.63	0.93	0.79	1.06	1.55
Control (n = 4)	7.89 ± 1.10 (5.89–10.8)	1.95 ± 0.64 (0.78–3.73)	0.89 ± 0.33 (0.53–1.89)	1.02 ± 0.16 (0.68–1.46)	0.13 ± 0.03 (0.05–0.23)

Abbreviations: MAD-S and MAD-M: severe and milder forms of MAD, respectively; asymptomatic: a case detected in the neonatal screening.
[*] P = 0.01 compared MAD-S with MAD-M.
[**] P = 0.04 compared MAD-M with control.
[†] P < 0.05 compared MAD-S with control.

Table 2
Acylcarnitine profiles in culture medium of fibroblasts from MAD patients incubated with unlabelled octanoic acid or myristic acid for 96 h.

Clinical type	Acylcarnitine ratio; mean ± SEM					
	Octanoic acid			Myristic acid		
	C8/C6	C8/C4	C8/C2	C14/C12	C14/C10	C14/C2
MAD-S (n = 3)	6.67 ± 3.63 (2.06–13.8)	1.20 ± 0.24 (0.81–1.63)	3.01 ± 1.92[*] (1.02–6.87)	3.66	3.03	4.42
MAD-M (n = 10)	2.73 ± 0.42 (0.88–4.56)	0.93 ± 0.14 (0.31–1.85)	0.73 ± 0.15[**] (0.14–1.38)	0.72	0.24	0.45
Asymptomatic (n = 1)	4.58	1.46	2.15	–	–	–
Control (n = 4)	2.10 ± 0.91 (0.69–4.71)	0.80 ± 0.25 (0.40–1.51)	0.15 ± 0.04 (0.08–0.24)	2.55	1.08	0.14

[*] P < 0.05 compared MAD-S with control.
[**] P < 0.05 compared MAD-M with control.

different FAOD (data not shown). Next, we determined if the acylcarnitine profiles can differentiate the clinical severity of MAD. When palmitic acid was loaded, a substantial accumulation of C16 was observed in the culture medium of cells with MAD-S, whereas the downstream C14, C12, C10, C8 or C2 did not show any increase. In contrast, C14, C12, C10 or C8 were elevated but C16 was lower in MAD-M compared to MAD-S (Fig. 1, right column). Therefore, the ratios of C16/C14, C16/C12, C16/C10, or C16/C8 were significantly higher in MAD-S than in MAD-M (Table 1). These findings suggest that oxidation of palmitic acid is inhibited in MAD-S, resulting in severely impaired production of acetyl-CoA.

Incubation with octanoic acid led to higher accumulation of C8-acylcarnitine in MAD-S cells compared to MAD-M (Fig. 1, left column). On the other hand, C2 was lower in MAD-S cells than that of MAD-M, as observed by palmitic acid. The ratio of C8/C2 showed significant increase in MAD-S compared to MAD-M (Table 2). Similarly, C8/C6 was higher in MAD-S (6.67 ± 3.63) than MAD-M (2.73 ± 0.42), though the difference was insignificant. Loading myristic acid to the MAD-S cells led to elevation of C14 acylcarnitine compared to control and MAD-M (Fig. 1, middle column). The ratios of C14/C12 and C14/C10 were also higher compared with those of MAD-M, as seen by palmitic or octanoic acid (Table 2). No difference was observed in the profile between infant and later childhood onset cases of MAD-M. The acylcarnitine profile was not associated with specific enzyme defect or gene; i.e. ETF-α, ETF-β and ETF-DH.

4. Discussion

MAD is a target disease of the neonatal screening using MS/MS. However, abnormalities on blood filter papers may not be detected in the stable condition of MAD-M or the presymptomatic stage, while the biochemical abnormalities are obviously observed in MAD-S and in the acute stage of MAD-M by GC/MS or MS/MS analyses. Actually, accurate biochemical diagnosis of MAD in presymptomatic stage is often difficult. Although genetic mutations of patients with MAD have been reported in various ethnic groups, almost all mutations do not seem to be associated with particular phenotype with a few exceptions [15], making it difficult to predict severity of the patients. The purpose of this study was to determine if the clinical severity in MAD can be evaluated using the in vitro probe acylcarnitine assay. Our data indicates that the in vitro probe acylcarnitine assay can clearly distinguish MAD-S from MAD-M.

An increase in C16 was observed exclusively in cells with MAD-S by loading palmitic acid, as opposed to a reduction in C14, C12, C10, C8 as well as C2. Loading octanoic acid or myristic acid also resulted in specific elevation of C8 or C14, respectively, in MAD-S. In contrast to MAD-S, the increase of C16 by palmitic acid was trivial in cells with MAD-M, whereas elevation of the downstream acylcarnitines C14, C12, C10 or C8 was larger compared to MAD-S. Similar to palmitic acid, such specific increase in C8 or C14 was barely detectable in cells with MAD-M upon incubation with octanotic or myristic acid, respectively. These results suggest that the milder enzyme deficiency in MAD-M allows the exogenous fatty acid substrates to process to some degree, resulting in elevation of downstream metabolites originated from loaded fatty acids. On the contrary, severe enzyme deficiency in MAD-S hampers to metabolize the loaded fatty acids to a shorter product, leading to a dramatic accumulation of the fatty acid corresponding to the substrates added. These hypotheses are consistent with significantly higher ratios between C16 and downstream acylcarnitines; i.e. C16/C14, C16/C12, C16/C12 or C16/C8, in cells with MAD-S compared to

MAD-M (Table 1). These findings strongly suggest that severity of MAD can be evaluated by quantitating the ratio between fatty acids loaded and the downstream metabolites.

Our results demonstrate that elevation of C16 by palmitic acid is one of the markers to characterize MAD-S. However, when compared with other FAODs, elevation of C16 is not specific to MAD-S, since palmitic acid also induced accumulation of C16 acylcarnitine in CPT2 deficiency without augmenting downstream metabolites (data not shown), which make acylcarnitine profile by palmitic acid in MAD-S look alike to CPT2 deficiency. However, a significant increase in C8 or C12 was observed by loading octanoic acid or myristic acid, respectively, in MAD-S, which was not observed in CPT-2 deficiency. This indicates that the enzyme activity for medium-chain fatty acids as well as long chain fatty acids is impaired in MAD-S, allowing MAD-S to be distinguished from CPT-2. However, cells from patients with respiratory chain defects may also show abnormalities similar to FAODs in the in vitro probe acylcarnitine assay [16,17], suggesting that the definitive diagnosis should be made in combination with acylcarnitine profiling and other laboratory tests, including genetic tests and enzymatic analysis.

In conclusion, our study indicates that the in vitro probe acylcarnitine assay using cultured fibroblasts loaded with various fatty acids allows us not just to distinguish MAD from other FAODs, but also clearly identify the severity of MAD. This strategy may be applied to evaluate the severity of the other metabolic diseases.

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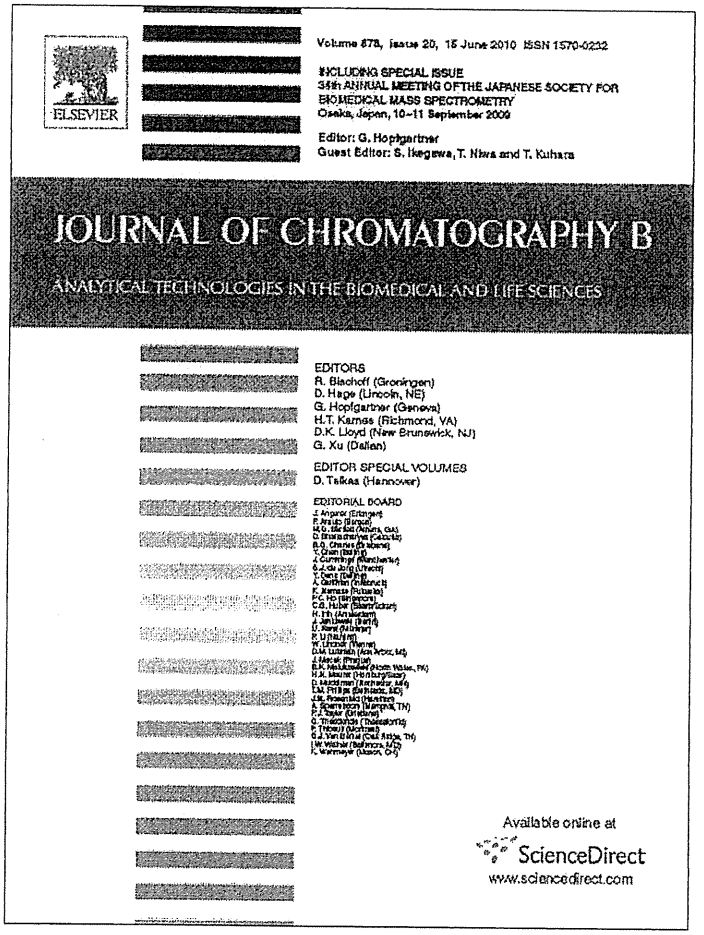

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Heat stress deteriorates mitochondrial β-oxidation of long-chain fatty acids in cultured fibroblasts with fatty acid β-oxidation disorders☆

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ABSTRACT

Mitochondrial fatty acids β-oxidation disorder (FAOD) has become popular with development of tandem mass spectrometry (MS/MS) and enzymatic evaluation techniques. FAOD occasionally causes acute encephalopathy or even sudden death in children. On the other hand, hyperpyrexia may also trigger severe seizures or encephalopathy, which might be caused by the defects of fatty acid β-oxidation (FAO). We investigated the effect of heat stress on FAO to determine the relationship between serious febrile episodes and defect in β-oxidation of fatty acid in children. Fibroblasts from healthy control and children with various FAODs, were cultured in the medium loaded with unlabelled palmitic acid for 96 h at 37 °C or 41 °C. Acylcarnitine (AC) profiles in the medium were determined by MS/MS, and specific ratios of ACs were calculated. Under heat stress (at 41 °C), long-chain ACs (C12, C14, or C16) were increased, while medium-chain ACs (C6, C8, or C10) were decreased in cells with carnitine palmitoyl transferase II deficiency, very-long-chain acyl-CoA dehydrogenase deficiency and mitochondrial trifunctional protein deficiency, whereas AC species from short-chain (C4) to long-chain (C16) were barely affected in medium-chain acyl-CoA dehydrogenase and control. While long-chain ACs (C12–C16) were significantly elevated, short to medium-chain ACs (C4–C10) were reduced in multiple acyl-CoA dehydrogenase deficiency. These data suggest that patients with long-chain FAODs may be more susceptible to heat stress compared to medium-chain FAOD or healthy control and that serious febrile episodes may deteriorate long-chain FAO in patients with long-chain FAODs.

1. Introduction

Tandem mass spectrometry (MS/MS) has been introduced to newborn screening for inherited metabolic diseases since early 1990s and become popular in diagnosis for mitochondrial fatty acid β-oxidation disorders (FAODs) [1,2]. Fatty acid β-oxidation (FAO) in mitochondria is a key energy generating process particularly under several conditions of metabolic stresses, like long fasting, prolonged exercises, infection or hyperpyrexia [3,4]. FAOD occasionally causes acute encephalopathy or even sudden death in children [5,6]. On the other hand, hyperpyrexia may also trigger some serious neurological symptoms, such as convulsion or acute encephalopathy [7,8]. There is a possibility that serious neurological symptoms related to hyperpyrexia are caused by transient or inherited defects of FAO. Recent reports suggest that long-chain fatty acid β-oxidation is inhibited during hyperpyrexia and that febrile episode may be one of potential reasons for the serious neurological events in influenza-associated encephalopathy (IAE) [9,10].

The β-oxidation of fatty acids is stepwise cycles and each turn of the cycle shortens the chain of fatty acid by two carbon atoms. There are multiple functional enzymes for each of fatty acids oxidative constituent step responsible for the oxidation of specific length chain fatty acids in mitochondria. If the enzymes involved in long-chain FAO such as carnitine palmitoyl transferase II (CPT2), very-long-chain acyl-CoA dehydrogenase (VLCAD), or mitochondrial trifunctional protein (MTP) are defective, long-chain ACs (C12, C14 or C16) will be accumulated [11–13]. If enzymes regulating medium-chain FAO are damaged, like medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, medium-chain ACs (C6, C8 or C10) will be accumulated [14]. In case electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH) are impaired, also called multiple acyl-CoA dehydrogenase (MAD) deficiency, a wide range

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Table 1
The values of R_p/R_c at different temperatures.

Groups (n = number of subjects)	Conditions	C4	C6	C8	C10	C12	C14	C16
Control	37 °C	1.00	1.00	1.00	1.00	1.00	1.00	1.00
(n = 6)	41 °C	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MCAD deficiency	37 °C	6.4 ± 1.4	3.2 ± 1.0	15.9 ± 3.4	3.1 ± 1.8	1.5 ± 0.2	1.3 ± 0.6	1.6 ± 0.5
(n = 4)	41 °C	3.4 ± 1.0*	2.9 ± 1.0	13.0 ± 4.0	3.5 ± 0.8	1.0 ± 0.4	1.1 ± 0.4	1.1 ± 0.2
CPT2 deficiency	37 °C	4.5 ± 0.6	2.3 ± 1.1	0.8 ± 0.5	0.5 ± 0.3	3.9 ± 0.4	6.3 ± 2.5	17.0 ± 2.2
(n = 2)	41 °C	5.0 ± 1.0	1.8 ± 0.1	2.0 ± 1.0	1.3 ± 0.1	12.2 ± 0.4*	9.9 ± 0.8	33.0 ± 3.6*
VLCAD deficiency	37 °C	1.0 ± 0.3	1.5 ± 0.5	2.3 ± 0.6	2.1 ± 0.6	6.0 ± 0.7	6.7 ± 2.2	2.6 ± 0.6
(n = 4)	41 °C	1.5 ± 0.5	1.0 ± 0.3	2.2 ± 0.2	2.7 ± 1.2	10.6 ± 1.5*	43.4 ± 5.6*	6.2 ± 0.8*
MTP deficiency	37 °C	1.3 ± 0.0	0.9 ± 0.1	0.9 ± 0.5	0.9 ± 0.4	8.2 ± 2.7	4.1 ± 1.5	8.7 ± 1.0
(n = 2)	41 °C	1.2 ± 0.2	2.1 ± 0.1*	1.2 ± 0.2	0.9 ± 0.3	25.7 ± 5.1*	17.9 ± 1.3*	19.2 ± 3.2*
MAD deficiency	37 °C	6.4 ± 0.8	10.1 ± 0.7	15.3 ± 0.1	15.2 ± 0.3	11.8 ± 1.4	13.8 ± 2.5	3.0 ± 0.3
(n = 2)	41 °C	1.3 ± 0.0*	1.7 ± 0.6*	4.4 ± 1.0*	4.7 ± 2.3*	12.3 ± 2.8	28.0 ± 0.8*	5.3 ± 1.1

Note: R_p represents the ratios of Cn/C2 (Cn: C4, C6, C8, C10, C12, C14, C14:1, C16) in patient cells. R_c represents the ratios of Cn/C2 in controls. C2–C16 represent specific length chain acylcarnitines, as shown in figure. The value of R_p/R_c represents fold change of patients compared to controls.
* $P<0.05$, values showed significant difference at 41 °C compared to 37 °C.

AC species from short to long-chain (C4–C16) will be elevated [15]. Furthermore, production of acetylcarnitine (C2), the final product of FAO cycle, will be suppressed in FAODs compared with that in normal control [16].

In the present study, we evaluated the effect of heat stress on impaired FAO, using MS/MS and cultured fibroblasts from several types of FAODs and healthy controls in order to determine the relationship between febrile episodes and defect in β-oxidation of fatty acid in children.

2. Materials and methods

2.1. Subjects

Human skin fibroblasts from 14 patients (passages 3–15) with various FAODs, which were diagnosed previously based on clinical and biochemical findings, plasma acylcarnitine profiles by MS/MS, as well as enzyme assay, were studied. These include 4 of VLCAD deficiency (def), each two of CPT2 def, MTP def, and MAD def, as well as 4 of MCAD def. Six cells (passages 3–16) from healthy volunteers were used as the control.

2.2. Cell culture

Cells were cultured in modified Eagle's minimal essential medium (MEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 2 mmol/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 10% FBS (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin (Nacalai Tesque) at 37 °C in a humidified 5% CO_2/95% air incubator until confluency.

2.3. In vitro probe acylcarnitine profiling assay in cultured cells under heat stress

Confluent cells in a 75 cm^2 flask were harvested by trypsinization (0.25%-Trypsin/1 mM-EDTA; Nacalai Tesque), then seeded onto 6-well microplates (35 mm i.d.; Iwaki) and re-cultured. When they reached confluence, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and cultured for 96 h in 1 mL of MEM with essential fatty acid–free BSA (0.4%; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabelled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, at 37 °C or 41 °C. After 96 h, AC profiling in the culture medium were analyzed by MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA).

2.4. MS/MS analysis

Methanol, acetonitrile, and formic acid were purchased from Wako (Osaka, Japan). The contents of an acylcarnitine (AC) reference standard kit (NSK-B, Cambridge Isotope Laboratories, Andover, USA): $^2[H]_9$-carnitine, $^2[H]_3$-acetylcarnitine, $^2[H]_3$-propionylcarnitine, $^2[H]_3$-butyrylcarnitine, $^2[H]_9$-isovalerylcarnitine, $^2[H]_3$-octanoylcarnitine, $^2[H]_9$-miristoylcarnitine, and $^2[H]_3$-palmitoylcarnitine, were diluted in methanol, and used as internal standard.

The sample preparation method for MS/MS analysis was described previously [17,18]. Briefly, 10 μL of the supernatant from culture medium was transferred to a 96-well microplate, and 200 μL methanol containing reference standard kit was added to each well. The aliquots were centrifuged at 1000 × g for 10 min, and then 150 μL of the supernatant was dried under a nitrogen stream, and butylated with 50 μL of 3N n-butanol–HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80% acetonitrile:water (4:1, v/v). The ACs in 10 μL of the aliquots were determined using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

2.5. Data statistical analysis

The results were expressed as mean ± SD from at least two independent experiments using the individual sample. The AC concentration was expressed as nmol/mg protein. Data were statistically analyzed by the one-way analysis of variance (ANOVA) and post hoc test for multiple group comparisons and Independent-samples T test for comparisons of two groups using SPSS version 11.5 software for Windows.

3. Results

3.1. Acylcarnitine profiling in various FAODs under heat stress

Incubation of cells from controls and patients deficient for MCAD, VLCAD, and MAD at 41 °C, increased C2 compared with 37 °C. The short-chain (C4), medium-chain (C6, C8 and C10), as well as long-chain ACs (C12, C14 or C16) were barely affected at 41 °C in control (Fig. 1A) and MCAD def (Fig. 1B). In contrast to MCAD def or control, long-chain ACs (C16 and/or C14 and C12) were increased at 41 °C in the cells from long-chain FAODs; CPT2 def (Fig. 1C), VLCAD def (Fig. 1D), and MTP def (Fig. 1E). Furthermore, while long-chain ACs (C12–C16) was elevated, short to medium-chain ACs (C4–C10) were significantly reduced at 41 °C in cells from MAD def (Fig. 1F).

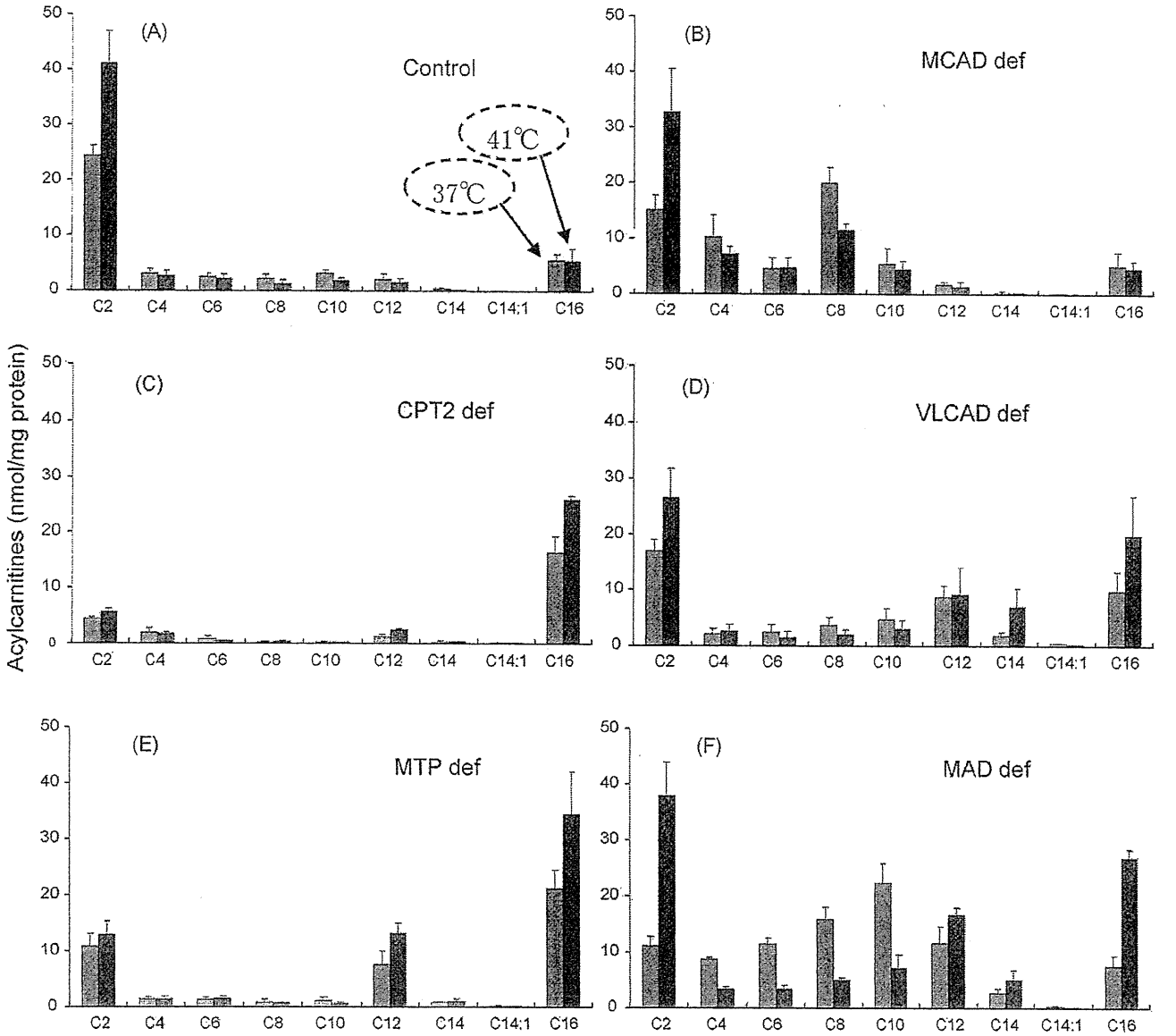

Fig. 1. AC profiles in supernatant of cells cultured with palmitate in various FAODs at 37 or 41 °C. ▒ : 37 °C; ▒ : 41 °C. A, Control; B, MCAD def (medium-chain acyl-CoA dehydrogenase deficiency); C, CPT2 def (carnitine palmitoyl transferase II deficiency); D, VLCAD def (very-long-chain acyl-CoA dehydrogenase deficiency); E, MTP def (mitochondrial trifunctional protein deficiency); F, MAD def (multiple acyl-CoA dehydrogenase deficiency). *Abbreviations*: C2, acetylcarnitine; C4, butyrylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C12, dodecanoylcarnitine; C14, myristoylcarnitine; C16, palmitoylcarnitine.

3.2. *The values of ratios of R_p/R_c at different conditions*

We calculated the ratios of R_p/R_c, which represent comparison of Cn/C2 (Cn: C4, C6, C8, C10, C12, C14, C14:1, C16) between patients (R_p) and controls (R_c) at different experimental conditions (Table 1). The value of R_p/R_c did not show any changes in MCAD deficient cells at different temperatures. R_p/R_c in long-chain ACs (C12, C14, C16) showed dramatic increase at 41 °C compared to 37 °C in cells from CPT2, VLCAD, and MTP deficiency. On the other hand, R_p/R_c from short to medium-chain (C4, C6, C8, C10) were lower whereas those from C14 was elevated at 41 °C compared to 37 °C in cells with MAD def.

4. Discussion

The present study evaluated the effect of heat stress, one of the most common metabolic stresses in children, on defective mitochondrial FAO to determine the relationship between febrile episodes and impaired FAO. We previously reported that accumulation of long-chain ACs was significantly enhanced at 41 °C compared with 37 °C in VLCAD-deficient cells [19]. Consistent with this observation, our current study showed that incubation of cells from patient deficient for CPT2, VLCAD or MTP at high temperature deteriorates long-chain FAO compared to physiological temperature. CPT2, VLCAD, and MTP are membrane-bound enzymes, located at inner-membrane of mitochondria and worked together towards β-oxidation of long-chain fatty acids [20]. Our results suggest that long-chain FAODs, such as deficiency for CPT2, VLCAD, as well as MTP, are susceptible to high temperature, which may be associated with metabolic crisis of these patients when they suffer from high fever. In contrast, short or medium-chain FAO was barely affected by heat stress. These data indicates that the effect of heat stress on FAO is different between long-chain ACs and short/medium-chain ACs. Consistent with these findings,

long-chain FAO was impaired whereas short and medium-chain FAO were facilitated by high temperature in patient cells lacking MAD, an enzyme involved in short to long-chain FAO. The data suggest that the electron transfer process by ETF and ETFDH for the flavin-containing dehydrogenases in long-chain FAO may be impaired at higher temperature in MAD deficiency without deteriorating medium-chain FAOs. While enzymes involved in long-chain FAO, such as VLCAD, TFP and CPT2 are bound to inner-membrane of mitochondria, MCAD and SCAD that catalyze medium and short-chain FAO are located in the mitochondrial matrix. These findings suggest that heat stress may selectively impair membrane-bound protein in contrast to those in the matrix. The underlying mechanism responsible for the differential effect of high temperature on ETF or ETFDH activity remains to be determined, but interaction of various dehydrogenases with ETF/ETFDH at differential locations may also partially be responsible for diverse effect on heat lability.

Previous reports suggest that impaired fatty acid β-oxidation may be responsible for influenza-associated encephalopathy (IAE), one of the life-threatening diseases resulting from influenza virus infection in children [9,10]. Other report also showed a decreased thermal stability of CPT2 variants in IAE patients during hyperpyrexia [21]. However, lack of definitive evidence explaining the mechanism responsible for the IAE resulting from deficiency of FAO makes it difficult to prove this association. Our data implies that impairment of mitochondrial FAO as a consequence of hyperpyrexia may be one of the mechanisms responsible for IAE.

In conclusion, our study suggests that patients with long-chain FAODs may be more susceptible to heat stress compared to medium-chain FAODs or healthy controls. Serious febrile episodes may further deteriorate long-chain FAO in FAODs. The underling pathogenic mechanism involved in impaired FAO by various stresses associated with life-threatening neurological episodes should be determined in future studies.

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Simple and rapid analytical method for detection of amino acids in blood using blood spot on filter paper, fast-GC/MS and isotope dilution technique

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ABSTRACT

A simple and rapid method for quantitative analysis of amino acids, including valine (Val), leucine (Leu), isoleucine (Ile), methionine (Met) and phenylalanine (Phe), in whole blood has been developed using GC/MS. In this method, whole blood was collected using a filter paper technique, and a 1/8 in. blood spot punch was used for sample preparation. Amino acids were extracted from the sample, and the extracts were purified using cation-exchange resins. The isotope dilution method using $^{2}H_{8}$-Val, $^{2}H_{3}$-Leu, $^{2}H_{3}$-Met and $^{2}H_{5}$-Phe as internal standards was applied. Following propyl chloroformate derivatization, the derivatives were analyzed using fast-GC/MS. The extraction recoveries using these techniques ranged from 69.8% to 87.9%, and analysis time for each sample was approximately 26 min. Calibration curves at concentrations from 0.0 to 1666.7 μmol/l for Val, Leu, Ile and Phe and from 0.0 to 333.3 μmol/l for Met showed good linearity with regression coefficients = 1. The method detection limits for Val, Leu, Ile, Met and Phe were 24.2, 16.7, 8.7, 1.5 and 12.9 μmol/l, respectively. This method was applied to blood spot samples obtained from patients with phenylketonuria (PKU), maple syrup urine disease (MSUD), hypermethionine and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), and the analysis results showed that the concentrations of amino acids that characterize these diseases were increased. These results indicate that this method provides a simple and rapid procedure for precise determination of amino acids in whole blood.

1. Introduction

Free amino acids in whole blood reflect the state of amino acid metabolism; consequently, amino acids in blood or serum are monitored in patients with inborn errors of amino acid metabolism [1–4]. The filter paper technique is widely used for sampling whole blood for analysis of amino acids, acylcarnitines and fatty acids due to its superior features in sampling, transportation and sample retention compared with serum samples [1,5,6].

Tandem mass spectrometry (MS/MS) [1,3], high performance liquid chromatography (HPLC) [2], liquid chromatography mass spectrometry (LC/MS) [7], gas chromatography (GC) [8] and gas chromatograph mass spectrometry (GC/MS) [5,9] methods have been reported for quantitative analysis of amino acids in whole blood using the filter paper technique. In the aforementioned methods, MS/MS is commonly used for screening of inborn errors of metabolism because of the short analysis time [3]. Conversely, HPLC, LC/MS, GC and GC/MS methods have been applied to quantitative analysis due to their performance in chromatographic separation. In particular, the GC/MS method offers exceptional chromatographic separation, detailed mass spectral analysis and low ion suppression.

For these reasons, GC/MS methods for the analysis of amino acids in blood have been developed. In sample preparation process, amino acids are extracted using cation-exchange resins [10–12] after proteins are removed from blood samples using sulphosalicylic acid [10,13,14] and picric acid [12,14] treatments. Extracted amino acids are derivatized for GC/MS analysis. If organic solvent treatment is used for the removal of proteins, the sample goes directly to derivatization following the drying procedure [5,8,15–17]. For the derivatization procedure, trimethylsilylation [4,18,19], tert-butyldimethylsilylation [20,21], esterifcation-acylation [5,16], and alkyl chloroformation [8,9,22–25] have been reported and those methods were summarized by Knapp [26] and Blau and Halket [27]. For quantitative calculation, the absolute calibration method is widely used but the isotope dilution method was used to improve the accuracy [10,17,28].

In this study, we have developed a method for analysis of amino acids in blood using the filter paper technique and GC/MS. For easy and simple sample preparation, a commercially available kit for analysis of amino acids was used; after sample purification

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on cation-exchange resins and propyl chloroformate derivatization were performed, the treated samples were analyzed using the fast-GC/MS method to shorten the analysis time. To improve the accuracy of the results, the isotope dilution technique was also applied for quantitative analysis.

For the validation of this method, phenylalanine, leucine, isoleucine, valine and methionine were chosen as target amino acids in consideration of current Japanese neonatal mass screening, in which phenylalanine, leucine, and methionine are measured for detection of phenylketonuria (PKU), maple syrup urine disease (MSUD), and homocystinuria (HCY), respectively.

This method was successfully applied to the analysis of amino acids in blood spot samples obtained from patients suffering from PKU, MSUD, hypermethioninemia and even neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD).

2. Experimental

2.1. Chemicals

$^{2}H_8$-valine ($^{2}H_8$-Val), $^{2}H_3$-leuicine ($^{2}H_3$-Leu), $^{2}H_3$-methionine ($^{2}H_3$-Met) and $^{2}H_5$-phenylalanine ($^{2}H_5$-Phe) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) to be used as internal standards. A mixture of the d-labeled amino acids was prepared at a concentration of 1 μmol/l in methanol. The solution mixtures of valine (Val), leucine (Leu), isoleucine (Ile), methionine (Met) and phenylalanine (Phe) were prepared at concentrations of 0.5, 1, 5, 10 and 50 μmol/l by diluting a stock solution of the amino acid mixture (200 μmol/l; EZ:faast™) with 0.05 N HCl solution.

2.2. Preparation

Whole blood samples were absorbed on a filter paper (ADVANTEC PKU, Tokyo, Japan). After drying at room temperature, one 1/8 in. punch from each blood spot was transferred into a 1.5 ml-vial, and 100 μl each of the internal standard mixture and 0.05 N HCl solution was added to the vial. The mixture was vortexed for 10 min, allowed to stand for 40 min and then vortexed a second time for an additional 10 min. The obtained supernatant was prepared following the procedure described in the Phenomenex EZ:faast™ amino acid analysis kit for GC/MS (Phenomenex Inc., CA, US) with the exception of the addition of the internal standard mixture to the solution instead of novaline.

2.3. GC/MS measurement

The blood sample analysis was performed on a gas chromatograph coupled to a quadrupole mass spectrometer (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan) equipped with an automatic injection system (AOC-20i+s) and a split/splitless injection port. The analytical conditions are shown in Table 1. A short capillary column (10 m × 0.25 mm I.D.) was used. The data acquisition interval was set to 0.2 s to collect more than eight data points for each of the observed GC peaks along the GC peaks [15,29].

2.4. Method validation

2.4.1. Recovery of preparation

A control experiment was performed to evaluate the extraction recovery of the amino acids from a blood spot sample; the amounts of amino acids recovered were assumed to be equal to those of the labeled amino acids. Whole blood from healthy control, which contained amino acids within the normal concentration ranges, was spotted onto filter paper, and the blood spot (1/8 in.) was punched. Internal standard (0.1 nmol each of $^{2}H_8$-Val, $^{2}H_3$-Leu, $^{2}H_3$-Met and $^{2}H_5$-Phe) was added directly to each punch, and they were dried as spiked samples. The punches were treated following the preparation procedure shown in Section 2.2, with the exception that the internal standard mixture was added. For the blank test, five blank samples that did not contain a blood spot punch were prepared in 1.5 ml-vials following the preparation procedure (Section 2.2). The extraction recovery was calculated by dividing the peak areas of spiked samples by those of blank samples for $^{2}H_8$-Val, $^{2}H_3$-Leu, $^{2}H_3$-Met and $^{2}H_5$-Phe.

Table 1
Analytical conditions for GC/MS.

Gas Chromatography	
Injection volume	1.0 μl
Injection mode	Split (1:15)
Injection temp.	280 C
Column oven	110 C—(30 C/min)—320 C (0 min)
Carrier gas	He
Flow control mode	70.2 cm/s (linear velocity)
Total Flow	21.8 ml/min
Column Flow	1.18 ml/min
Purge Flow	3.0 ml/min
Mass spectrometry	
Interface temp.	280 C
Ion box temp.	200 C
Ionization voltage	70 eV
Emission current	150 μA
Data acquisition rate	0.2 s
Monitor ion (*m/z*)	
1.15–2.09 min	72,74,80,86,89, 116, 124, 130, 133, 158, 166, 172, 175
2.09–2.76 min	61,64, 190, 193
2.76–3.76 min	120, 126, 148, 154, 190, 196

2.4.2. Calibration curve

Blank and standard mixtures of Val, Leu, Ile, Met and Phe (0.5, 1, 5, 10 and 50 nmol/l) were analyzed and used to construct calibration curves according to a least-squares linear regression equation. Because one blood spot punch corresponds to 3 μl of whole blood, the concentrations of the standard mixtures (0.0, 0.5, 1, 5, 10 and 50 nmol/l in analysis) were converted to those of amino acids in whole blood (0.0, 16.7, 33.3, 166.7, 333.3 and 1666.7 μmol/l). The concentrations were varied from 0.0 to 1666.7 μmol/l for Val, Leu, Ile and Phe and from 0.0 to 333.3 μmol/l for Met. A concentration of 33.0 μmol/l of $^{2}H_8$-Val, $^{2}H_3$-Leu, $^{2}H_3$-Met and $^{2}H_5$-Phe was added as an internal standard for corresponding non-labeled amino acids, and $^{2}H_3$-Leu was substituted for isotope-labeled Ile.

2.4.3. Method detection limit

The method detection limits were determined by analyzing five blood punches from a healthy control, which contained amino acids within the normal concentration ranges, and multiplying the standard deviation of the mean by the appropriate Student's *t*-value for the 99% confidence level using the appropriate degrees of freedom.

2.5. Healthy control and patient sample analysis

Blood spot samples were obtained from 33 normal controls and analyzed by the methods outlined in Sections 2.2 and 2.3. The mean values and the standard deviations obtained from 33 normal controls were calculated. The cut-off value was defined as the mean plus three standard deviations.

Blood spot samples were obtained from 5 patients with disorders diagnosed by the MS/MS method and clinical symptoms for PKU, MSUD and hypermethionine NICCD. To validate the new method, each patient's sample was analyzed to determine Val, Leu, Ile, Met and Phe concentrations, which were compared with the cut-off values.

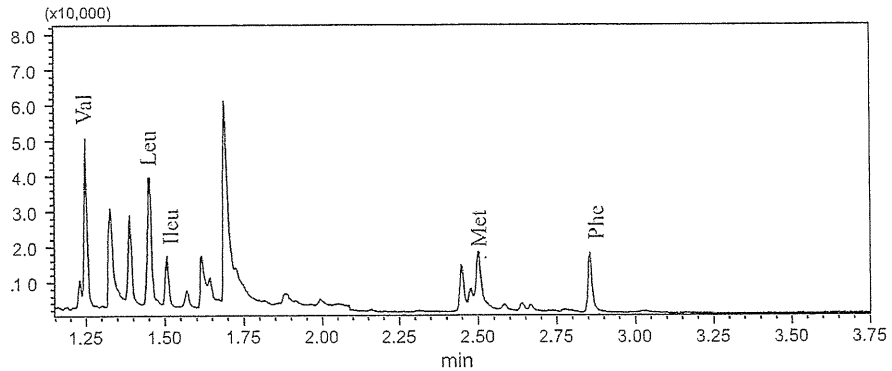

Fig. 1. Total ion chromatogram of a blood spot sample. A total ion chromatogram of a blood spot sample obtained from a healthy control is shown. Val = Valine, Leu = Leucine, Ile = Isoluecine, Met = Methionine and Phe = Phenylalanine.

3. Results

3.1. GC/MS measurement

Fig. 1 shows the total ion chromatogram of a blood spot sample obtained from a healthy control. Retention times of Val, Leu, Ile, Met and Phe were 1.25, 1.46, 1.51, 2.50 and 2.86 min, respectively. All target amino acids were separated by selective ion monitoring (*m/z* 116, 172, 130, 190 and 190 for Val, Leu, Ile, Met and Phe, respectively) without overlapping component peaks (Fig. 2).

3.2. Method validation

3.2.1. Recovery of preparation

The extraction recoveries varied from 69.8% to 87.9%, as shown in Table 2. The repeatabilities for blank samples and control samples were <4.4% and 14.1% (RSD%, $n = 5$), respectively.

Table 2
Recovery and repeatability.

	Blank (n = 5)		Punch of blood (n = 5)		
Compound	Mean[a]	%RSD	Mean[b]	%RSD	Recovery[c]
$^2H_8$-Valine	3575	4.4%	2496	12.3%	69.8%
$^2H_3$-Leucine	6449	2.9%	4515	13.5%	70.0%
$^2H_3$-Methionine	615	2.5%	541	13.4%	87.9%
$^2H_5$-Phenylalanine	2096	3.6%	1655	14.1%	79.0%

[a] The internal standard mixture (0.1 nmol) was added directly to each punch of the blood spot sample obtained from a healthy control. The dried punch was treated following the preparation procedure shown in Section 2.2. The means of the indicated peak areas are shown.

[b] For the blank test, five blank samples that did not contain a blood spot punch were prepared in the 1.5 ml vial were prepared following the preparation procedure (Section 2.2). The means of the indicated peak areas are shown.

[c] Recovery = peak area (punch of blood)/peak area (blank).

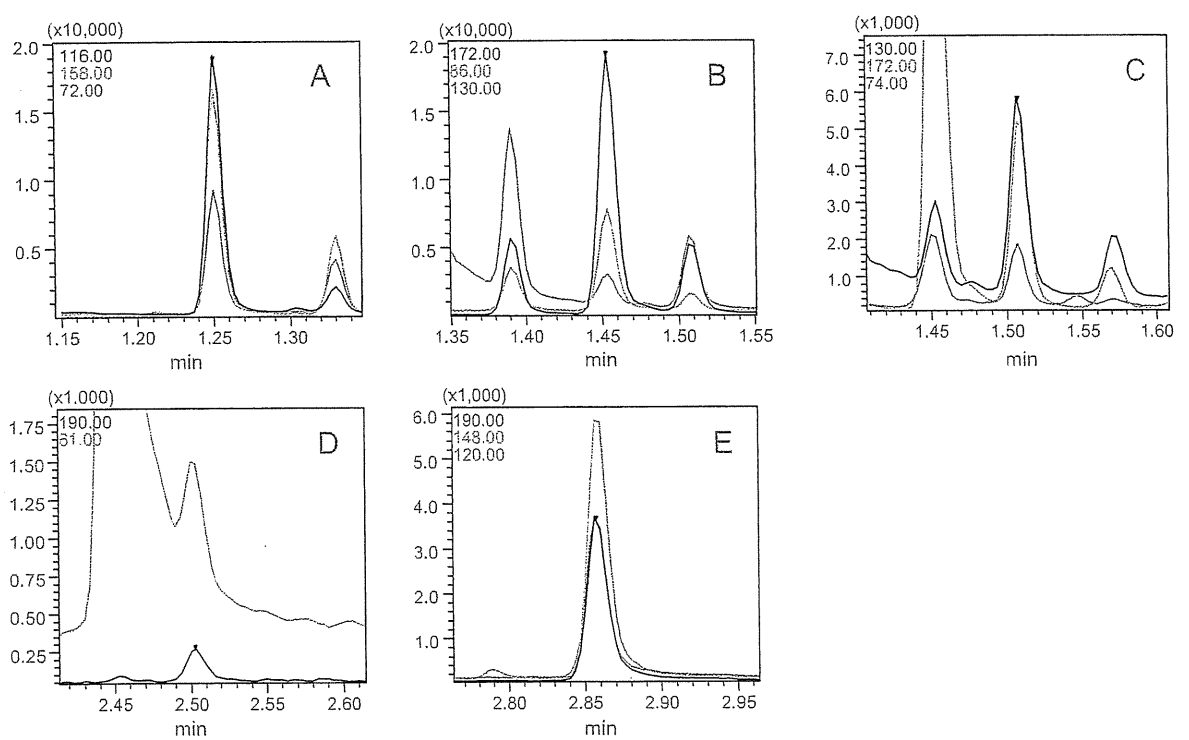

Fig. 2. Selected ion monitoring of a blood spot sample. Selected ion monitoring of amino acids in a blood spot sample obtained from a healthy control is shown. All target amino acids were separated by selected ion monitoring without overlapping of component peaks. A: Valine, *m/z* 116, 129.7 μmol/l; B: Leucine, *m/z* 172, 104.7 μmol/l; C: Isoleucine, *m/z* 130, 41.3 μmol/l; D: Methionine, *m/z* 190, 13.3 μmol/l and E: Phenylalanine, *m/z* 190, 63.0 μmol/l.

Table 3
Calibration curves.

Compound	Equations	R^2 [a]
Valine	$y = 1.41x + 2.60 \times 10^{-2}$	1.000
Leucine	$y = 1.08x + 0.10 \times 10^{-1}$	1.000
Isoluecine	$y = 0.73x + 2.76 \times 10^{-2}$	1.000
Methionine	$y = 0.91x + 4.62 \times 10^{-1}$	0.999
Phenylalanine	$y = 1.12x + 5.17 \times 10^{-2}$	1.000

The concentrations varied from 0.0 to 1666.7 μmol/l for Val, Leu, Ile and Phe and from 0.0 to 333.3 μmol/l for Met. A concentration of 33.0 μmol/l of $^2H_8$-Val, $^2H_3$-Leu, $^2H_3$-Met and $^2H_5$-Phe was added as an internal standard for corresponding non-labeled amino acids; $^2H_3$-Leu was also substituted for isotope labeled Ile. The concentrations of 0.0, 0.5, 1, 5, 10 and 50 nmol/l were converted to 0.0, 16.7, 33.3, 166.7, 333.3 and 1666.7 μmol/l of amino acids in whole blood.

[a] Correlation coefficient.

3.2.2. Calibration curve

The correlation coefficients for Val, Leu, Ile and Phe were 1.000 at concentrations from 0.0 to 1666.7 μmol/l, and the correlation coefficient for Met was 0.999 at concentrations from 0.0 to 333.3 μmol/l as shown in Table 3.

3.2.3. Method detection limit

Results obtained from 5 blood spot samples of a healthy control are shown in Table 4. The repeatabilities for the method (%RSD, $n = 5$) were <5.0% for the evaluated amino acids. The method detection limits ranged from 1.5 (Met) to 24.2 μmol/l (Val).

3.3. Healthy control and patient sample analysis

Table 5 shows the analytical results obtained for blood spot samples from 5 patients suffering from PKU, MSUD and hypermethionine NICCD and from 33 healthy controls. The concentrations of Val, Leu, Ile, Leu + Ile, Met and Phe in the 33 healthy controls were lower than the cut-off values.

4. Discussion

Methods using GC or GC/MS for analysis of amino acids in whole blood, which used blood spot samples on filter papers, have been reported [5,9]. In these previous reports, a sample punch 8 mm in diameter was used; in this study, we used a punch of 1/8 in., which allowed for more sample punches to be taken from the same spot if re-analysis or other biochemical tests are required.

Amino acids were extracted from punches of blood spots with a mixture of methanol, which was used as the solvent for the internal standard, and 0.05 N HCl (1:1, v/v). During the solvent extraction, most of protein could be removed similar as organic solvent treatment [4,5,8,16]. Cation-exchange resins were used to extract amino acids from the solvent. Adsorption efficiency of aliphatic amino acids, such as Phe, on ion exchange resins was not decreased by the non-polar extraction solvent, which was approximately 50% methanol. The extraction process showed excellent recovery and repeatability (79.0% and 14.1% %RSD) for $^2H_5$-Phe. For the other amino acids, the recoveries were more than 69.8%, and the repeatability was <13.5% (%RSD, $n = 5$) without internal standard correction. These results indicate that not only non-aliphatic amino acids, such as Val, Leu, Ile and Met, but also aliphatic amino acids, such as Phe, can be quantitatively extracted using this method.

Amino acids are usually analyzed by GC/MS after derivatization of the amine and carboxylic functional groups. Various derivatization methods were evaluated as described in Section 1. In those methods, residual water in the sample does not interfere with propyl chloroformation and this derivatization method may be highly preferable in biological samples [9,24,25,30]. Additionally, derivatization time was shortened by using propyl chloroformate. Trimethylsilylation, which is commonly used in derivatization for GC/MS analysis, requires solvent dehydration via heating and longer derivatization reaction time (30–60 min). Conversely, propyl chloroformate is unaffected by water, and the reaction is complete within 1 min at room temperature. For these reasons, propyl chroloformation was applied to this study.

Amine and carboxylic functional groups are converted to carboxylicpropyl and propylester, respectively, by the propyl chloroformation derivatization, and the mass number of the molecular ion is increased by 128 u. In Leu, Met and Phe, mass spectra of the derivatives showed molecular ions at m/z 259, 277 and 293, respectively; however, a similar molecular ion was not detected in mass spectra of Ile. In addition, mass spectra of derivatives of Val, Leu, Ile, Met and Phe showed specific ions formed by loss of a C_3H_7COO fragment (87 u) from the molecular ion at m/z 158, 172, 172, 190 and 206, corresponding to the loss of this fragment, i.e. Val: 245 – 87, Leu: 259 – 87, Ile: 259 – 87, Met: 277 – 87 and Phe: 293 – 87 (data not shown). These results demonstrate that the evaluated amino acids were completely derivatized by this method.

A high linear velocity of 70.2 cm/s, which was generated by a short capillary column (10 m × 0.25 mm I.D.), and a fast oven temperature program of 30 °C/min were used to shorten GC/MS analysis time (Table 1). The GC/MS analysis cycle time was approximately 10 min; the Phe had the longest retention time (2.86 min). Under these conditions, all target amino acids were separated and selectively detected (Fig. 2). During 50 sample analyses, interference due to peak overlap did not occur. These results suggest that the solvent extraction and purification on the cation ion-exchange resins were appropriate to selectively separate the amino acids from sample contaminants, and these pre-analysis steps resulted in good chromatographic separation. This method reduces GC/MS analysis time by one-third to one-sixth compared to the conventional method (30–60 min) while retaining good chromatographic separation [9,20,31].

Table 4
Repeatability and method detection limits.

Compound	Concentrations for five blood punches[b] [μmol/l]					Mean	%RSD	MDLs[c]
	1	2	3	4	5			
Valine	129.7	145.7	140.7	135.3	139.3	138.1	4.3%	24.2
Leucine	104.7	114.0	113.7	108.0	113.0	110.7	3.7%	16.7
Isoluecine	41.3	45.3	44.7	40.7	44.7	43.3	5.0%	8.7
Leu + Ile[a]	146.0	159.3	158.3	148.7	157.7	154.0	4.0%	24.9
Methionine	13.3	12.7	12.3	12.7	13.0	12.8	3.0%	1.5
Phenylalanine	63.0	70.7	70.0	66.7	69.7	68.0	4.7%	12.9

[a] Total values of Leu (Leucine) and Ile (Isoluecine).

[b] The method detection limits were determined by analyzing five punches of the same blood spot from a healthy control, which contained amino acids within the normal concentration ranges.

[c] MDLs (Method detection limits) were determined by multiplying the standard deviation of the mean by the appropriate Student's t-value for the 99% confidence level using the appropriate degrees of freedom.

Table 5
Analytical results obtained for blood spot samples from 5 patients.

Disease		Val		Leu		Ile		Leu + Ile		Met		Phe	
		[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]
PKU[a]	1	110.7	0.40	69.8	0.34	40.6	0.36	110.3	0.36	9.4	0.17	***619.0***	***6.01***
	2	*189.3*	0.68	124.3	0.61	66.5	0,90	190.8	0.62	8.7	0.16	***201.6***	***1.96***
MSUD[b]	1	***360.3***	***1.29***	***2646.6***	***13.07***	***141.4***	***1.27***	***2788.0***	***9.08***	4.6	0.08	40.4	0.39
	2	***297.0***	***1.06***	***1017.4***	***5.02***	***257.0***	***2.30***	***1274.4***	***4.15***	6.6	0.12	43.7	*0.42*
NICDD[c]	1	178.1	0.64	91.6	0.45	49.9	0.45	141.5	0.46	***300.8***	***5.39***	***168.0***	***1.63***
Control[d]													
Mean		166.0	0.59	105.5	0.52	59.9	0.54	165.3	0.54	25.9	0.46	60.3	0.59
Cut-off		279.7	1.00	202.5	1.00	111.5	1.00	307.0	1.00	55.8	1.00	102.9	1.00

[a] Phenylketonuria.
[b] Maple syrup urine disease.
[c] Hypermethionine and neonatal intrahepatic cholestasis caused by citrin deficiency.
[d] 33 healthy controls.
[e] Ratio to cut-off value.

From these results, total analysis time was 80 min, which included a 60-min extraction time, a 10-min purification and derivatization time and a 10-min fast-GC/MS analysis time that included column cool-down. Although the extraction time was relatively long, it could be easily shortened by processing more samples in a batch. If 10 samples were processed as one batch, the analysis time for each sample would be only 6 min extraction time. In the reported results, total analysis time per sample with this method was 26 min and could be dramatically shortened compared to the conventional method.

The evaluated method was applied to amino acid analysis in a blood spot punch, and the method detection limits were determined (Table 3). The MDLs of Val, Leu, Ile, Leu + Ile, Met and Phe were lowered by factors of 5.96, 8.23, 14.95, 5.42, 5.97 and 16.20, respectively, compared with cut-off values (Table 5). The maximum concentrations were up to 11.56, 12.13, 12.82, 12.33, 37.2 and 7.98 times higher compared to cut-off values. These results show that this method can be applied to amino acids in whole blood at concentrations ranging from 0.18 (Leu + Ile) to 7.98 (Phe) of cut-off values, which should be sufficient for a biochemical test for inborn errors of amino acid metabolism [3,32].

Deng and Deng [5] reported that amino acids in blood were measured using the blood filter paper technique similar to our method. Amino acids were derivatized by n-buthanol and trifluoroacetic acid. The repeatability was lower than 5%, which was similar to our results, but the detection limits were lower than ours. The supposed reason is that the diameter of the punch (8 mm) was larger than ours (1/8 in.). However, the linearity of calibration curves ranged from 0.988 to 0.998, which were not good compared to ours. As those results, isotope dilution method is superior to non-isotope method for a quantitative calculation.

The method developed in this study was applied to five blood spot samples obtained from patients with inborn errors of amino acid metabolism, including PKU, MSUD and hypermethionine NICCD (Table 5). PKU is characterized by an increasing concentration of phenylalanine in the blood. Our results showed that the concentration of Phe was 1.96 and 6.01 times higher than the cut-off value. In maple syrup urine disease (MSUD), Leu, Ile, and Val accumulate in the blood. Our results showed the concentration of Leu was 13.07 and 5.02 times higher and that of Ile was 1.27 and 2.03 times higher than the cut-off values. In hypermethionine NICCD, phenylalanine, galactose, methionine or threonine increase in the blood. In this study, samples from a hypermethionine NICCD patient exhibited a concentration of Met that was 5.39 times higher than the cut-off value. These results show that this method can be applied to the chemical diagnosis of inborn errors of amino acid metabolism through the determination of the concentrations of the amino acids that are characteristically higher when these diseases are present.

The MS/MS method is superior to other methods in analysis time (only 2 min) and less expensive due to the application of flow injection as a method of sample introduction in MS/MS. For these reasons, the MS/MS method is widely applied to neonatal screening for inborn errors of amino acid, organic acid and fatty acid metabolism [1,5,6]. However, the GC/MS method has several aspects that are superior to the MS/MS method. In the MS/MS method, Leu and Ile are detected at the same *m/z* value without chromatographic separation and cannot be separated and determined individually. Ion-suppression effects due to co-eluting matrix components are not negligible in the MS/MS method, which prevents precise determination of analytes [33–35]. GC/MS can be used to avoid possible matrix effects that are detected by the MS/MS because the GC/MS can separate target compounds from the sample matrix with high chromatographic resolution. Electron ionization (EI)-GC/MS is also more resistant to ion-suppression than electrospray ionization-MS/MS. The characteristic mass spectral pattern obtained by EI can provide the mass numbers in the target compound, which do not overlap with other substances, so target compounds can be detected selectively. These advantages indicate that the GC/MS method is more appropriate for analyses in which lower analytical errors are required, such as for therapy monitoring and for specific patient diagnosis (e.g. moderate hyper-excretions or not an acute episode). The GC/MS method is necessary as a back-up method for MS/MS, especially as a precise quantitative method. In clinical laboratories, GC/MS is already widely used for various analyses, such as for organic acids in urine and for very long chain fatty acids in plasma that are indicative of an inborn error of metabolism [19,36–38]; thus, this method of amino acid analysis using GC/MS would be useful for those laboratories[9].

5. Conclusion

This new method enables simple, rapid and precise analysis for determination of amino acids in whole blood using GC/MS. It was successfully applied to 5 patients with 3 types of amino acid disorders, providing similar concentration levels to those reported using other methods.

Our study demonstrated the feasibility of routine biochemical test of amino acids using this method. Therefore, further studies to expand other amino acids should be meaningful in order to apply this method to routine biochemical tests for inborn errors of amino acid metabolism.

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ステロイド抵抗性の難治性アトピー性皮膚炎として加療されていたビオチン欠乏の1例

Biotin deficiency misdiagnosed as steroid-resistant atopic dermatitis

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Key words：ビオチン，食物アレルギー，特殊ミルク，マルチプルカルボキシラーゼ欠損症，尿中有機酸分析

Abstract

8ヶ月の男児。出生直後から家族の判断によりアレルギー用特殊ミルクが使用されていた。生後3か月から湿疹が出現し，難治性アトピー性皮膚炎としてステロイドと特殊ミルクを変更しながら加療されていた。眼瞼，口唇，肛門周囲の粘膜皮膚移行部の難治性湿疹と脱毛，及び特殊ミルクにビオチンが含まれていないことから尿中有機酸分析を行い，ビオチン欠乏と診断した。ビオチン欠乏による湿疹は，ビオチン補充により早期に改善するものの診断がつきにくい。特殊ミルクを飲んでいる乳児に難治性湿疹を見た場合，ビオチン欠乏を鑑別にあげる必要がある。

はじめに

水溶性ビタミンの一つであるビオチンは多くの食品に含まれており，腸内細菌叢でも合成されるため，通常の食物摂取をしている時は不足することはない。一方，ミルクアレルギー等で使用する治療用の特殊ミルクにはビオチンがほとんど含まれておらず，単独での使用ではビオチン欠乏を生じる危険がある[1)2)]。日本では2003年にビオチンが食品添加物として保健機能食品には認められるようになったが，特別用途食品である特殊ミルクや乳児用調製粉乳には現在でも使用できない[3)]。

ビオチンは4つのカルボキシラーゼに共通する補酵素として働き，ビオチン欠乏では二次性マルチプルカルボキシラーゼ欠損症（MCD）として種々の症状を呈する（図1）。特徴的な臨床所見として眼瞼周囲や口唇，会陰部などの粘膜皮膚移行部にみられる難治性湿疹，脱毛がある。鑑別すべき先天性のMCDには，ホロカルボキシラーゼ合成酵素(HCS）欠損症，ビオチニダーゼ欠損症がある[4)]。

ビオチン欠乏の診断には尿中有機酸分析が有用である。4つのカルボキシラーゼの酵素活性低下を反映した異常代謝産物（図1）の排泄増加により診断が可能であり[4)]，中でも3-OH-isovalerateはビオチン欠乏の早期診断に鋭敏なマーカーであると報告されている[5)]。その他，血中アシルカルニチン分析でのイソバレリルカルニチン（C5OH）の上昇，血中と尿中のビオチン濃度低下も診断に役立つ[4)]。

島根大学小児科では，1995年以降，尿中有機酸分析を行っているが，特殊ミルクに伴う

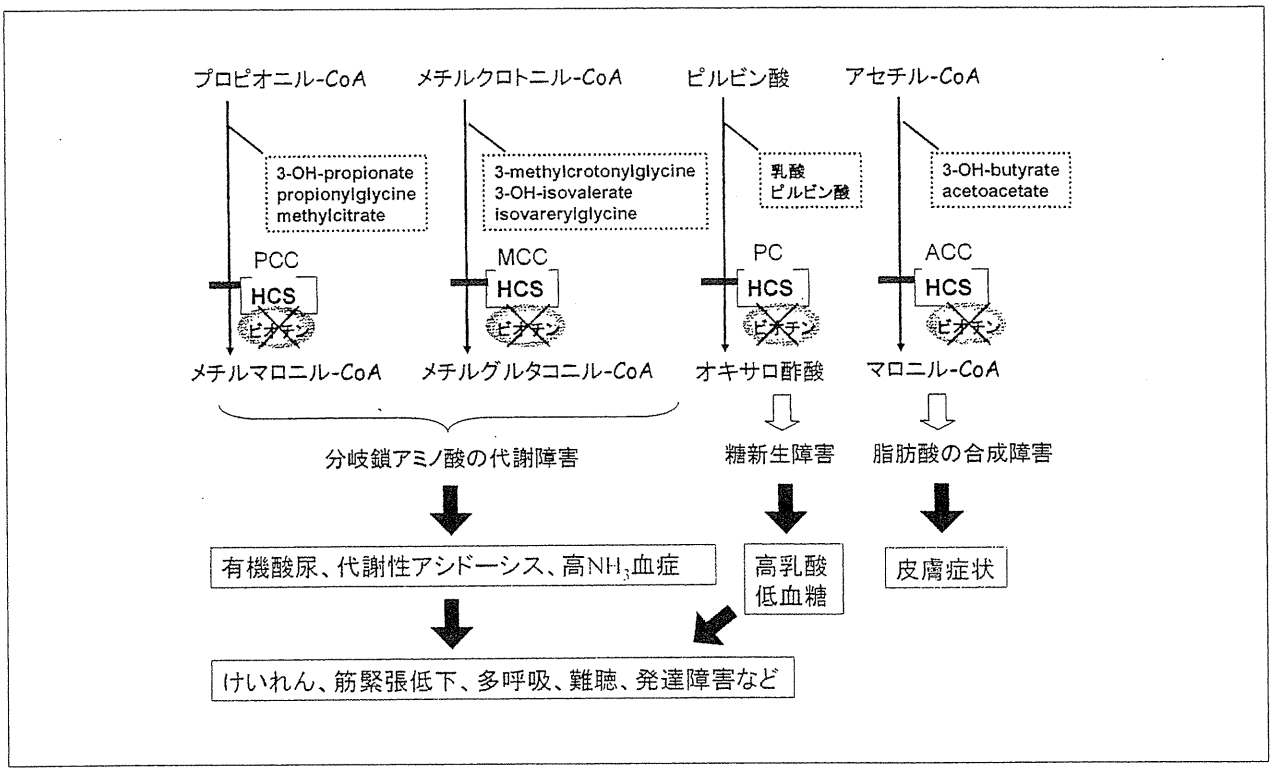

図1　マルチプルカルボキシラーゼ欠損症の代謝マップと臨床症状
ビオチンは4つのカルボキシラーゼに共通するHCSの補酵素として働いているため，ビオチン欠乏ではこれら4つの経路がすべて障害される。点線内の異常代謝産物が尿中に増加することにより診断が可能となる。HCS:ホロカルボキシラーゼ合成酵素，PCC:プロピオニル-CoAカルボキシラーゼ，MCC:メチルクロトニル-CoAカルボキシラーゼ，PC:ピルビン酸カルボキシラーゼ，ACC:アセチル-CoAカルボキシラーゼ。

二次性ビオチン欠乏の症例が近年増加傾向にあるように思われる。今回我々は，アレルギー用特殊ミルク使用中にステロイド抵抗性の難治性湿疹として加療をうけていたビオチン欠乏症の1例を報告する。

1. 症例

患者：8ヶ月，男児
主訴：難治性湿疹
家族歴：母親がアトピー性皮膚炎のため民間療法を行っている。姉（6歳）がアトピー性皮膚炎および食物アレルギーのため，外来治療を行っている。
現病歴：周産期歴に異常なし。家族の判断により出生直後からミルフィー®のみの哺乳が行われていた。1ヶ月健診では異常を指摘されていないが，その後，生後3ヶ月頃より顔面，頭部，体幹部と眼瞼，口唇，肛門周囲の粘膜皮膚移行部に湿疹がみられるようになった。近医で難治性アトピー性皮膚炎として加療されたが改善がなく，家族の判断でミルクをニューMA1®に変更した。生後5ヶ月に皮膚症状が悪化したため近医に入院し，消毒や抗生剤の使用，外用薬の調整，さらにミルクをエレメンタルフォーミュラ®に変更したが改善しなかった。また，生後8ヶ月時点で，離乳食は開始できていなかった。生後8ヶ月に加療目的のため当院のアレルギー専門外来に紹介受診となった。
身体所見：身長74.7cm(+1.8 S.D)，体重7.7kg(-0.8 S.D)。寝返りは可能だが，坐位は不

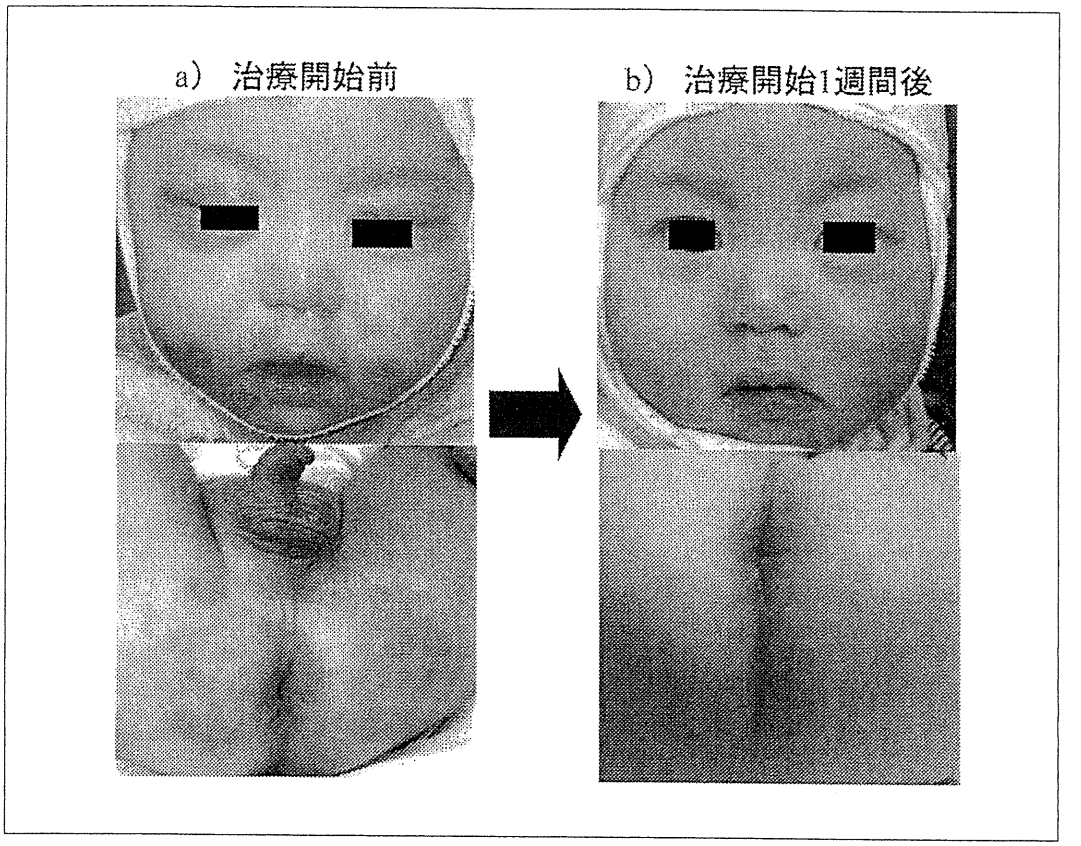

図2　治療前後における皮膚所見の変化

安定で，はいはいはできなかった。皮膚所見（図2a）では，眼瞼，口唇，肛門周囲にびらん性紅斑を認め，頭頸部に貨幣状湿疹，脂漏性湿疹，浸潤性紅斑を，臀部，体幹部に発赤疹を認めた。その他，脱毛と爪がもろい所見がみられた。

一般検査所見（図3）：一般検査では軽度の鉄欠乏性貧血と肝逸脱酵素の上昇を認めた。アレルギー検査ではIgE，RASTの上昇はみられないが，好酸球が16%と高値を示した。微量元素である亜鉛は軽度低下していた。

特殊検査所見（図4）：尿中有機酸分析ではMCDでみられる特徴的な排泄物の増加を認め，血液ろ紙による血中アシルカルニチン分析ではC5OHの増加と遊離カルニチンの低下を認めた。ビオチンによる治療開始1週間後でもビオチン血中濃度は低値であった。一方，治療前の尿中ビオチン濃度は正常範囲であった。

経過：眼瞼，口唇および肛門周囲の粘膜皮膚移行部の湿疹と脱毛がみられたこと，ビオチンを含まないアレルギー用ミルクを使用していたこと，尿中有機酸分析と血中アシルカルニチン分析による代謝スクリーニングの結果からビオチン欠乏による皮膚炎と診断した。直ちにビオチン1mg/日，カルニチン300mg/日の内服を開始し，治療1週間で皮膚症状の明らかな回復が認められた（図2b）。以降，軽度から中等度のアトピー性皮膚炎はあるが，離乳食をすすめながら1歳10ヶ月にビオチン，カルニチンの内服を中止した後も悪化はなくステロイドでコントールできている。1歳で喃語のみと言語発達の軽度の遅れがあったが，1歳6ヶ月の時点では運動言語とも

発達の遅れを認めなかった。

2. 考察

本症例はステロイド抵抗性の難治性アトピー性皮膚炎として加療が継続され，ビオチン欠乏の診断が遅れた症例であった。ミルクアレルギーを否定できず特殊ミルクを継続していたことが問題であり，離乳食を生後8ヶ月まで開始することができなかったことも症状を悪化させたと思われる。ビオチンが含まれていない特殊ミルクを使用し，眼瞼周囲，口唇，会陰部の粘膜皮膚移行部にみられる難治性湿疹をみた際にはビオチン欠乏を考える必要がある。

ビオチン欠乏による難治性湿疹は，ビオチン内服後1～2週間程度で改善する場合が多い[6)~8)]。尿中有機酸分析等の特殊検査に時間がかかる場合には，尿と血液ろ紙，血清を保存した上でビオチン内服による治療的診断を行うことも検討するべきである。本症例ではビオチン投与後に軽度発達遅滞が改善しており，ビオチン欠乏が影響していた可能性も否定できないと考えている。

本症例ではHCS欠損症の遺伝子解析は行っていないが，新生児期の血液ろ紙血を用いて後方視的に行った血中アシルカルニチン分析でC5OHの上昇を認めないこと，血中のビオチニダーゼ酵素活性（兵庫県立大学の渡邊敏明先生により測定）が保たれていたことより，後天性のMCDと考えられた。湿疹が四肢末端にはみられないこと[9)]，特殊ミルクには亜鉛が添加されていることより[1)]，亜鉛の補充は行わなかった。

また，特殊ミルクによる二次性ビオチン欠乏では，本症例のようにカルニチン欠乏を合併することがあり注意を要する。遊離カルニチンが低下する原因には二つある。一つはビオチンと同様にカルニチンも特殊ミルクに含まれていないこと[3)]，もうひとつは蓄積した

表1　一般検査所見

WBC	16,500	/μl	Na	138	mEq/l
(Eos	16%)		K	4.5	mEq/l
RBC	435万	/μl	Cl	101	mEq/l
Hg	12.2	g/dl	Ca	9.8	mg/dl
Plt	32.1万	/μl	BS	87	mg/dl
TP	5.6	g/dl			
Alb	3.8	g/dl	IgE	33.7	KU/l
T-Bil	0.2	mg/dl	RAST	陰性	
AST	32	IU/l	(卵、牛乳、米、小麦、大豆、カゼイン)		
ALT	51	IU/l	Fe	40	/dl
LDH	309	IU/l	TIBC	358	/dl
Alp	560	IU/l	フェリチン	12.1	ng/ml
CK	125	IU/l	亜鉛	60	μg/dl
BUN	11.4	mg/dl		(基準値 65-100)	
Cre	0.17	mg/dl			

表2　特殊検査所見

尿中有機酸分析（島根大学小児科）
治療前
methycitrate、3-OH-isovalerate
3-methylcrotonylglycine、isovalerylglycine の排泄増加

血液ろ紙における血中アシルカルニチン分析（島根大学小児科）
治療前
C5OH 3.83 μM/l (-1.0) 、遊離カルニチン 8.8 μM/l (10-60)

ビオチン濃度（兵庫県立大学　渡邊敏明先生）

尿中ビオチン濃度	治療前	26.3 μg/g･Cre (4.0-25.0)
	治療後3ヶ月後	14.0 μg/g･Cre
血清freeビオチン濃度	治療後1週間後	0.3ng/ml (0.4-1.1)
	治療後3ヶ月後	0.8ng/ml