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

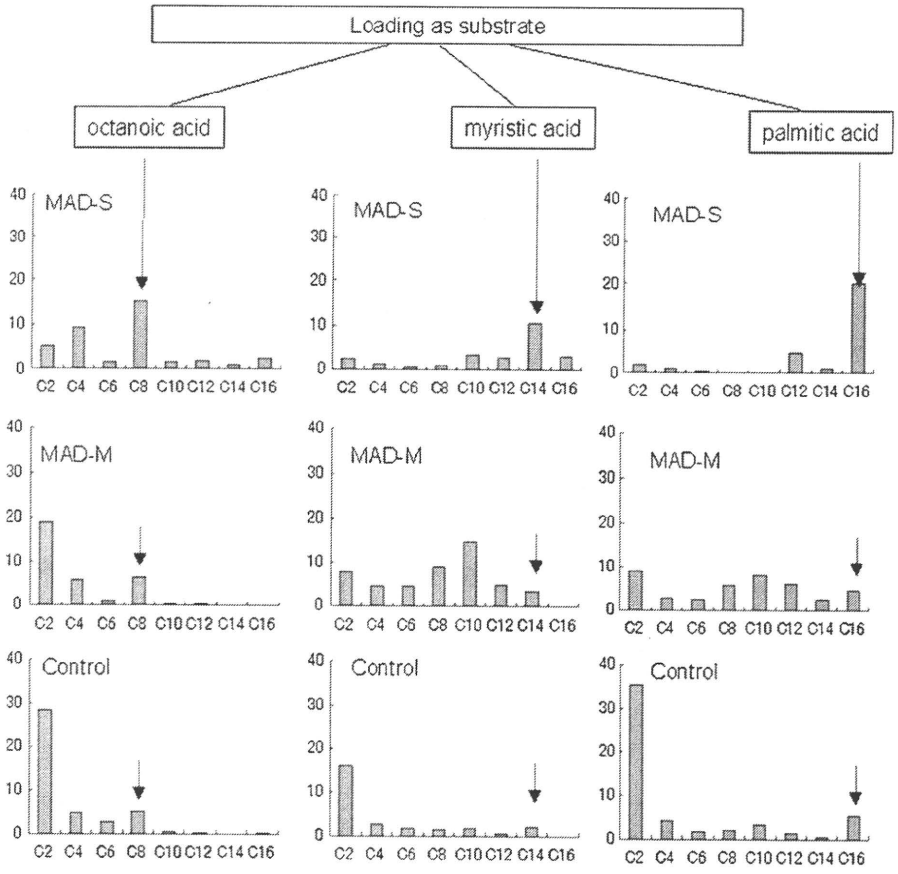


Fig. 1. The acylcarnitine profiles of fibroblasts from MAD-S and MAD-M. Arrows indicate loaded fatty acids; octanoic acid, myristic acid or palmitic acid. The *Y* axis represents values of acylcarnitines expressed as nmol/mg protein/96 h.

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.

Acknowledgements

We are grateful to Mrs. Ito and Furui for their technical assistance. This study was partly supported by grants from The Ministry of Health, Labor and Welfare of Japan, from the Ministry of Education, Culture, Sports, Science and Technology, and from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References

[1] K. Tanaka, N. Gregersen, A. Ribes, J. Kim, S. Kolvraa, V. Winter, H. Eiberg, G. Martinez, T. Deufel, B. Leifert, R. Santer, B. Francois, E. Pronicka, A. Laszlo, S. Kmoch, I. Kremensky, L. Kalaydjicva, I. Ozalp, M. Ito, Pediatr. Res 41 (1997) 201.
[2] Y. Tamaoki, M. Kimura, Y. Hasegawa, M. Iga, M. Inoue, S. Yamaguchi, Brain Dev. 24 (2002) 675.
[3] F.E. Frerman, S.I. Goodman, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 4517.
[4] N. Gregersen, B.S. Andresen, M.J. Corydon, T.J. Corydon, R.K. Olsen, L. Bolund, P. Bross, Hum. Mutat. 18 (2001) 169.
[5] S. Yamaguchi, T. Orii, Y. Suzuki, K. Maeda, M. Oshima, T. Hashimoto, Pediatr. Res. 29 (1991) 60.
[6] M.A. al-Essa, M.S. Rashed, S.M. Bakheet, Z.J. Patay, P.T. Ozand, J. Perinatol. 20 (2000) 120.
[7] K.G. Sim, J. Hammond, B. Wilcken, Clin. Chim. Acta 323 (2002) 37.
[8] J.G. Okun, S. Kolker, A. Schulze, D. Kohlmuller, K. Olgemoller, M. Lindner, G.F. Hoffmann, R.J. Wanders, E. Mayatepek, Biochim. Biophys. Acta 1584 (2002) 91.
[9] K. Giak Sim, K. Carpenter, J. Hammond, J. Christodoulou, B. Wilcken, Mol. Genet. Metab. 76 (2002) 327.
[10] C. Vianey-Saban, P. Divry, M. Brivet, M. Nada, M.T. Zabot, M. Mathieu, C. Roe, Clin. Chim. Acta 269 (1998) 43.
[11] D.S. Roe, C. Vianey-Saban, S. Sharma, M.T. Zabot, C.R. Roe, Clin. Chim. Acta 312 (2001) 55.
[12] S.E. Olpin, S. Clark, B.S. Andresen, C. Bischoff, R.K. Olsen, N. Gregersen, A. Chakrapani, M. Downing, N.J. Manning, M. Sharrard, J.R. Bonham, F. Muntoni, D.N. Turnbull, M. Pourfarzam, J. Inherit. Metab. Dis. 28 (2005) 533.
[13] D.S. Roe, B.Z. Yang, C. Vianey-Saban, E. Struys, L. Sweetman, C.R. Roe, Mol. Genet. Metab. 87 (2006) 40.
[14] J.J. Shen, D. Matern, D.S. Millington, S. Hillman, M.D. Feezor, M.J. Bennett, M. Qumsiyeh, S.G. Kahler, Y.T. Chen, J.L. Van Hove, J. Inherit. Metab. Dis. 23 (2000) 27.
[15] Y. Yotsumoto, Y. Hasegawa, S. Fukuda, H. Kobayashi, M. Endo, T. Fukao, S. Yamaguchi, Mol. Genet. Metab. 94 (2008) 61.
[16] K.G. Sim, K. Carpenter, J. Hammond, J. Christodoulou, B. Wilcken, Metabolism 51 (2002) 366.
[17] J.J. Gargus, K. Boyle, M. Bocian, D.S. Roe, C. Vianey-Saban, C.R. Roe, J. Inherit. Metab. Dis. 26 (2003) 659.

Journal of Chromatography B, 878 (2010) 1669–1672

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

Hong Li[a,b,*], Seiji Fukuda[a], Yuki Hasegawa[a], Jamiyan Purevsuren[a], Hironori Kobayashi[a], Yuichi Mushimoto[a], Seiji Yamaguchi[a,**]

[a] Department of Pediatrics, Shimane University School of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan
[b] Department of Pediatrics, The Affiliated Hospital of Ningxia Medical University, 804 Sheng-li-jie, Yinchuan, Ningxia 750004, China

ARTICLE INFO

Article history:
Received 31 October 2009
Accepted 30 January 2010
Available online 11 February 2010

Keywords:
Fatty acids β-oxidation disorder
Heat stress
Tandem mass spectrometry
Acylcarnitine profiling

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

☆ This paper was presented at the 34th Annual Meeting of the Japanese Society for Biomedical Mass Spectrometry, Osaka, Japan, 10–11 September 2009.

* Corresponding author at: Department of Pediatrics, Shimane University School of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan. Tel.: +81 853 20 2216; fax: +81 853 20 2215.
** Corresponding author. Tel.: +81 853 20 2216; fax: +81 853 20 2215.
E-mail addresses: nxlihong@163.com (H. Li), seijiyam@med.shimane-u.ac.jp (S. Yamaguchi).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.01.046

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).

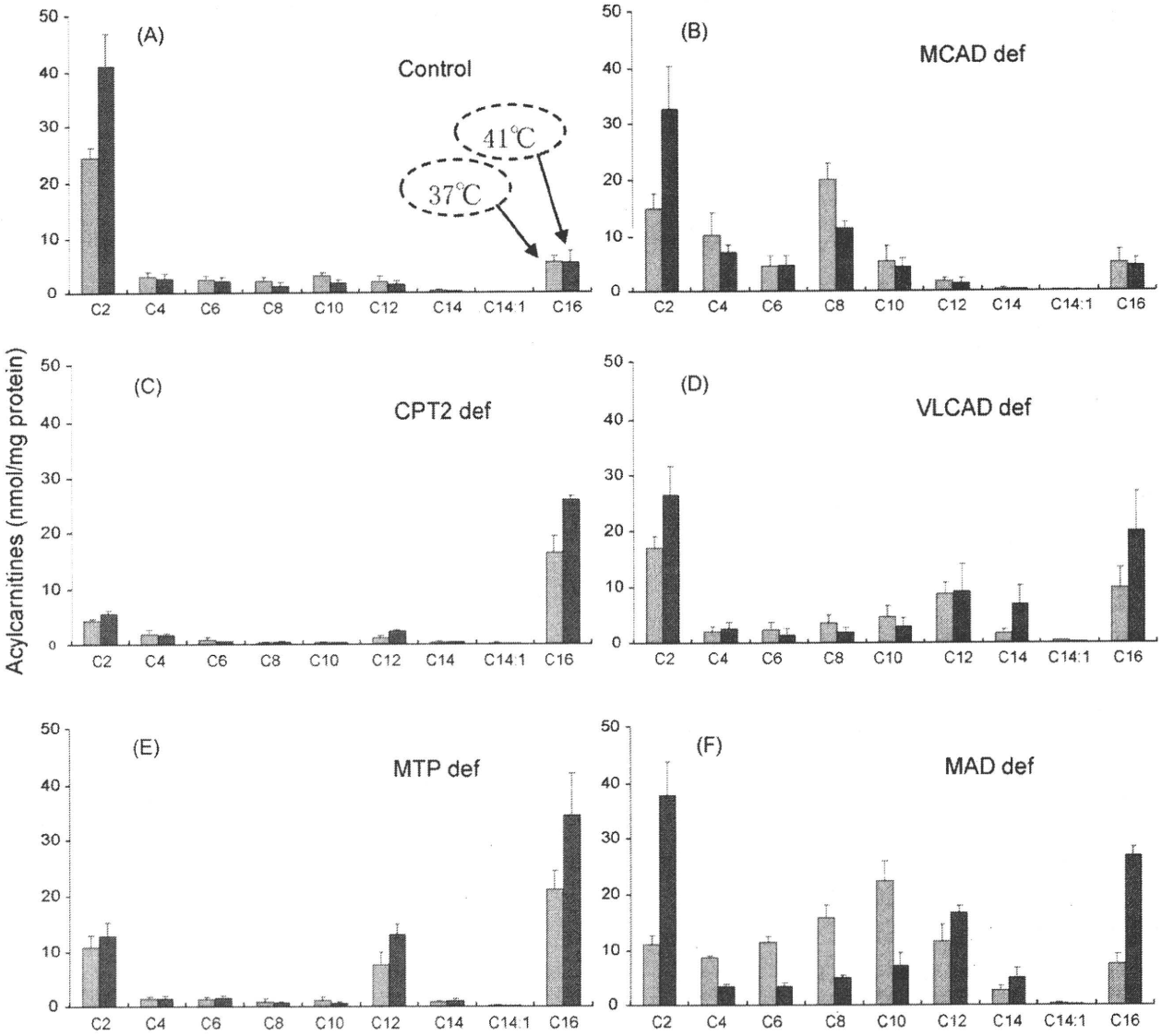


Fig. 1. AC profiles in supernatant of cells cultured with palmitate in various FAODs at 37 or 41 °C. ▒ : 37 °C; ■ : 41 °C. A, Control; B, MCAD def (medium-chain acyl-CoA dehydrogenase deficiency); C, CPT2 def (carnitine palmitoyl transferase II deficiency); D, VLCAD def (very-long-chain acyl-CoA dehydrogenase 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 C*n*/C2 (C*n*: 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.

Acknowledgments

We are grateful to Ms. Yuka Ito, Toyomi Esumi, Midori Furui, and Nana Tomita for their technical assistance. This study was partly supported by grants from the Ministry of Health, Labour and Welfare of Japan, from the Ministry of Education, Culture, Sports, Science and Technology, and from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References

[1] P. Rinaldo, D. Matern, M.J. Bennett, Annu. Rev. Physiol. 64 (2002) 477.
[2] B. Wilcken, V. Wiley, J. Hammond, K. Carpenter, N. Engl. J. Med. 348 (2003) 2304.
[3] S. Ghisla, Eur. J. Biochem. 271 (2004) 459.
[4] U. Spiekerkoetter, M. Lindner, R. Santer, M. Grotzke, M.R. Baumgartner, H. Boehles, H. de Klerk, I. Knerr, H.G. Koch, B. Plecko, W. Roschinger, K.O. Schwab, D. Scheible, F.A. Wijburg, J. schocke, E. Mayatapek, U. Wendel, J. Inherit. Metab. Dis. 32 (2009) 488.
[5] J.B. Lundemose, S. Kolvraa, N. Gregersen, E. Christensen, M. Gregersen, J. Clin. Pathol: Mol. Pathol. 50 (1997) 212.
[6] A.W. Strauss, C.K. Powell, D.E. Hale, M.M. Anderson, A. Ahuja, J.C. Brackett, H.F. Sims, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 10496.
[7] R.G. Ahmed, Int. J. Dev. Neurosci. 23 (2005) 549.
[8] T. Togashi, Y. Matsuzono, M. Narita, T. Morishima, Virus Res. 103 (2004) 75.
[9] Y. Chen, H. Mizuguchi, D. Yao, M. Ide, Y. Kuroda, Y. Shigematsu, S. Yamaguchi, M. Yamaguchi, M. Kinoshita, H. Kido, FEBS Lett. 579 (2005) 2040.
[10] J. Purevsuren, Y. Hasegawa, H. Kobayashi, M. Endo, S. Yamaguchi, Brain Dev. 30 (2008) 520.
[11] D.S. Roe, B.Z. Yang, C. Vianey-Saban, E. Struys, L. Sweetman, C.R. Roe, Mol. Genet. Metab. 87 (2006) 40.
[12] D.S. Roe, C. Vianey-Saban, S. Sharma, M.T. Zabot, C.R. Roe, Clin. Chim. Acta 312 (2001) 55.
[13] R.K.J. Olsen, S.E. Olpin, B.S. Andresen, Z.H. Miedzybrodzka, M. Pourfarzam, B. Merinero, F.E. Frerman, M.W. Beresford, J.C.S. Dean, N. Cornelius, O. Andersen, A. Oldfors, E. Holme, N. Gregersen, M. Douglass, D.M. Turnbull, A.A.M. Morris, Brain 130 (2007) 2045.
[14] J.G. Okun, S. Kolker, A. Schulze, D. Kohlmuller, K. Olgemoller, M. Lindner, Biochim. Et. Biophys. Acta 1584 (2002) 91.
[15] K.G. Sim, J. Hammond, B. Wilcken, Clin. Chim. Acta 323 (2002) 37.
[16] M.A. Nada, W.J. Rhead, H. Sprecher, H. Schulz, C.R. Roe, J. Biol. Chem. 270 (1995) 530.
[17] A. schulze, M. Lindner, D. Kohlmuller, K. Olgemoller, E. Mayatepek, G.F. Hoffmann, Pediatrics 111 (2003) 1399.
[18] S. Nomachi, T. Nakajima, M. Sakurada, N. Ota, M. Fukushi, K. Yano, U.G. Jensen, Ann. Rep. Sapporo City Inst. Public Health 34 (2007) 37.
[19] H. Li, S. Fukuda, Y. Hasegawa, H. Kobayashi, Y. Mushimoto, S. Yamaguchi, Brain Dev., 2009. [Epub ahead of print].
[20] U. Garg, M. Dasouki, Clin. Biochem. 39 (2006) 315.
[21] D. Yao, H. Mizuguchi, M. Yamaguchi, H. Yamada, J. Chida, K. Shikata, H. Kido, Hum. Mutat. 29 (2008) 718.

Journal of Chromatography B, 878 (2010) 3113–3118

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

Shuichi Kawana*, Katsuhiro Nakagawa, Yuki Hasegawa, Seiji Yamaguchi

Department of Pediatrics, Shimane University School of Medicine, 89-1 En-ya-cho, Izumo, Shimane 693-8501, Japan

ARTICLE INFO

Article history:
Received 28 May 2010
Accepted 20 September 2010
Available online 29 September 2010

Keywords:
Gas chromatograph–Mass spectrometer
Amino acids
Propyl chloroformate
Blood spot sample

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

* Corresponding author. Tel.: +81 75 823 1410; fax: +81 75 841 9326.
E-mail addresses: s_kawana@shimadzu.co.jp (S. Kawana), nk@shimadzu.co.jp (K. Nakagawa), yukirin@med.shimane-u.ac.jp (Y. Hasegawa), seijiyam@med.shimane-u.ac.jp (S. Yamaguchi).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.09.015

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.

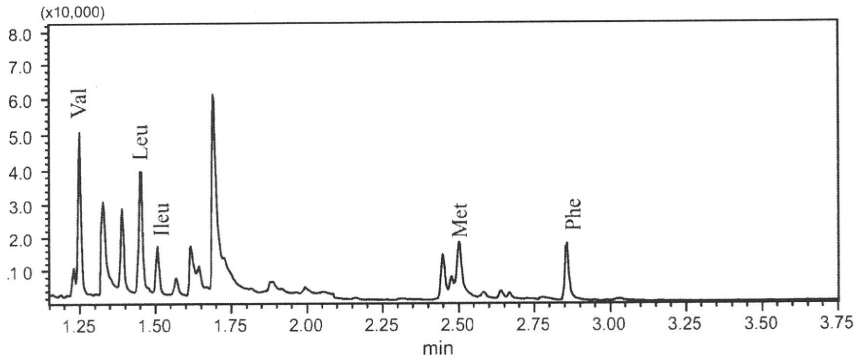


Fig. 1. Total ion chromatogram of a blood spot sample. A total ion chromatogram of a blood spot sample obtained from a healthy control is shown. Val = Valine, Leu = Leucine, Ile = Isoluecine, Met = Methionine and Phe = Phenylalanine.

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).

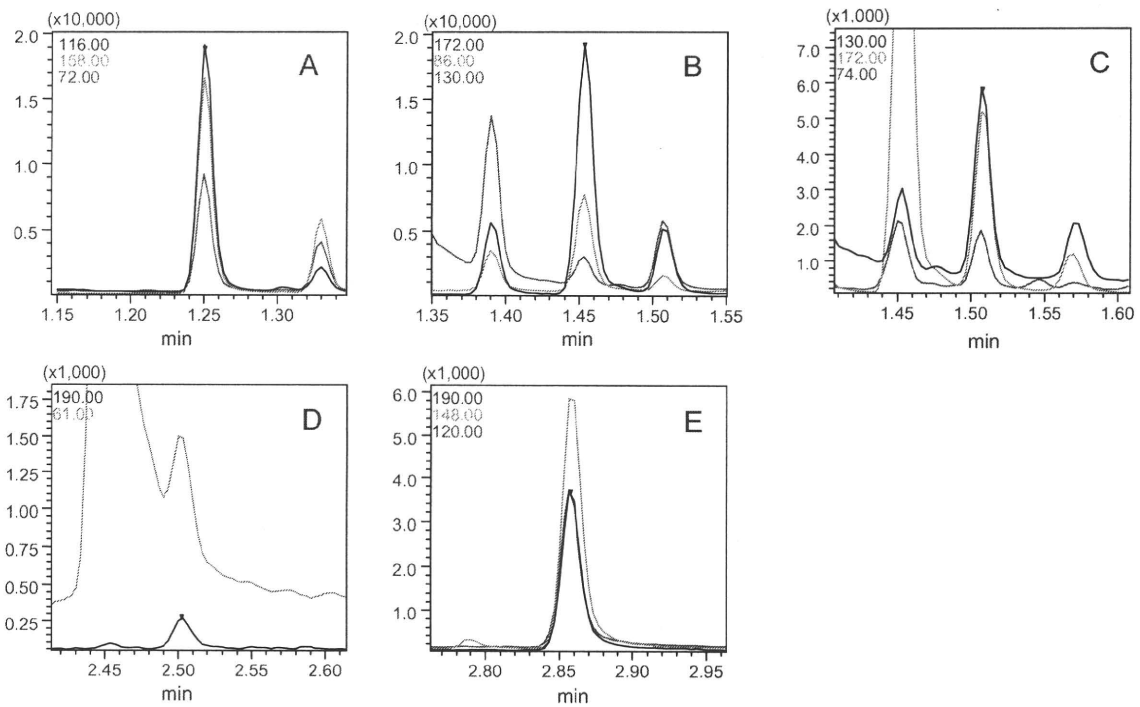


Fig. 2. Selected ion monitoring of a blood spot sample. Selected ion monitoring of amino acids in a blood spot sample obtained from a healthy control is shown. All target amino acids were separated by selected ion monitoring without overlapping of component peaks. A: Valine, *m/z* 116, 129.7 μmol/l; 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_190	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.

Acknowledgements

The authors express their appreciation to Dr. Novalina Lingga for helpful suggestions on the manuscript. This study was partly supported by grants from The Ministry of Education, Culture, Sports, Science, and Technology of Japan, from The Ministry of Health, Labor and Welfare of Japan.

References

[1] D.H. Chace, T.A. Kalas, E.W. Naylor, Clin. Chem. 49 (2003) 1797.
[2] A.A. Reilly, R. Bellisario, K.A. Pass, Clin. Chem. 44 (1998) 317.
[3] M.S. Rashed, M.P. Bucknall, D. Little, A. Awad, M. Jacob, M. Alamoudi, M. Alwattar, P.T. Ozand, Clin. Chem. 43 (1997) 1129.
[4] A. Jiye, J. Trygg, J. Gullberg, A.I. Johansson, P. Jonsson, H. Antti, S.L. Marklund, T. Moritz, Anal. Chem. 77 (2005) 8086.
[5] C. Deng, Y. Deng, J. Chromatogr. B 792 (2003) 261.
[6] H. Nishio, S. Kodama, S. Yokoyama, T. Matsuo, T. Mio, K. Sumino, Clin. Chim. Acta 159 (1986) 77.
[7] D. Oglesbee, K.A. Sanders, J.M. Lacey, M.J. Magera, B. Casetta, K.A. Strauss, S. Tortorelli, P. Rinaldo, D. Matern, Clin. Chem. 54 (2008) 542.
[8] P. Hušek, J. Chromatogr. B 669 (1995) 352.
[9] C. Deng, N. Li, X. Zhang, Rapid Commun. Mass Spectrom. 18 (2004) 2558.
[10] A.G. Calder, K.E. Garden, S.E. Anderson, G.E. Lobley, Rapid Commun. Mass Spectrom. 13 (1999) 2080.
[11] R.J.W. Truscott, D. Malegan, E. McCairns, B. Halpern, J. Hammond, R.G.H. Cotton, J.F.B. Mercer, S. Hunt, J.G. Rogers, D.M. Danks, Biomed. Mass Spectrom. 8 (1981) 99.
[12] H. Frank, A. Rettenmeier, H. Weicker, G.J. Nicholson, E. Bayer, Clin. Chim. Acta 105 (1980) 201.
[13] T.L. Perry, S. Hansen, Clin. Chim. Acta 25 (1969) 53.
[14] M.D. Armstrong, U. Stave, Metabolism 22 (1973) 549.
[15] J. Dallüge, R.J.J. Vreuls, D.J. van Iperen, M. van Rijn, U.A.Th. Brinkman, J. Sep. Sci. 25 (2002) 608.
[16] C. Deng, C. Shang, Y. Hu, X. Zhang, J. Chromatogr. B 775 (2002) 115.
[17] G.W. Lynes, M. Hjelm, J. Chromatogr. 562 (1991) 213.
[18] C. Deng, Y. Deng, B. Wang, X. Yang, J. Chromatogr. B 780 (2002) 407.
[19] I. Matsumoto, T. Kuhara, Mass Spectrom. Rev. 15 (1996) 43.
[20] T. Ohie, X.W. Fu, M. Iga, M. Kimura, S. Yamaguchi, J. Chromatogr. B 746 (2000) 63.
[21] I. MolnaÂr-Perl, Z.F. Katona, Chromatographia 51 (2000) S-228.
[22] T.G. Sobolevsky, A.I. Revelsky, I.A. Revelsky, B. Miller, V. Oriedo, J. Chromatogr. B 800 (2004) 101.
[23] P. Hušek, J. Chromatogr. 552 (1991) 289.
[24] J. Wang, Z. Huang, D.A. Gage, J.T. Watson, J. Chromatogr. A 663 (1994) 71.
[25] M. Vincenti, C. Minero, S. Lago, C. Rovida, J. High Resol. Chromatogr. 18 (1995) 359.
[26] D.R. Knapp, Handbook of Analytical Derivatization Reactions, John Wiley & Sons, Inc., New York, 1979.
[27] K. Blau, J.M. Halket (Eds.), Handbook of Derivatives for Chromatography, 2nd ed., John Wiley & Sons Ltd., Chichester, UK, 1993.
[28] M.W. Haymond, C.P. Howard, J.M. Miles, J.E. Gerich, J. Chromatogr. 183 (1980) 403.
[29] S. Kawana, K. Katsuhiro, Y. Hasegawa, H. Kobayashi, S. Yamaguchi, Clin. Chim. Acta 392 (2008) 34.
[30] T.G. Sobolevsky, A.I. Revelsky, B. Miller, V. Oriedo, E.S. Chernetsova, I.G. Revelsky, J. Sep. Sci. 26 (2003) 1474.
[31] T. Kuhara, J. Chromatogr. B 758 (2001) 3.
[32] R. Blau, M. Duran, M.E. Blaskovics, K.M. Gibson, Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, 2nd ed., Springer, New York, 2003.
[33] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[34] H.R. Liang, R.L. Foltz, M. Meng, P. Bennett, Rapid Commun. Mass Spectrom. 17 (2003) 2815.
[35] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[36] S.I. Goodman, S.P. Markey, Diagnosis of Organic Acidemias by Gas Chromatograpy–Mass Spectrometry, Alan R. Liss, Inc., New York, 1981.
[37] P. Vreken, A.E.M. van Lint, A.H. Bootsma, H. Overmars, R.J.A. Wanders, A.H. van Gennip, J. Chromatogr. B 713 (1998) 281.
[38] M. Kimura, T. Yamamoto, S. Yamaguchi, Tohoku J. Exp. Med. 188 (1999) 317.

＜診断へのアプローチ —first line 検査＞

アシドーシス，ケトーシス

深尾敏幸＊

はじめに

First line 検査としての血液ガス分析は血糖，血液ガス，アンモニアという 3 点セットのひとつであり，いわゆる急性発症型（けいれん，嘔吐，多呼吸，意識障害など脳炎や脳症を疑わせる症状）をとるタイプの代謝異常症のスクリーニングの必須項目である。またケトーシスの有無はアシドーシスの鑑別に重要であり，遊離脂肪酸とケトン体分画を一緒に測定する。

I 代謝性アシドーシス

一般には血液ガスは動脈採血であるが，よほどの循環不全がなければ，代謝疾患の評価には静脈血でもよい（通常の静脈採血での動脈血との pH，HCO_3^- の差が代謝異常症の診断に影響をあたえる可能性はきわめて少ない）。静脈血でもいいから検査しておくことが重要である。一般に，pH 7.30 未満，HCO_3^- 15 mmol/L 未満は代謝性アシドーシスが強いと考える。

代謝性アシドーシスは小児科においてよく認められる所見であり，先天代謝異常症がなくても，感染，異化亢進状態，脱水，組織低酸素状態などでも認められる。一方，代謝性アシドーシスをきたす先天代謝異常症は上述のような状態（感染，異化亢進など）で発症することも多い。代謝性アシドーシスの存在と評価は血液ガス分析を行うことが必須であり，病態の解釈には電解質，血糖，乳酸ピルビン酸，ケトン体，遊離脂肪酸，アンモニアとの同時測定することが望ましい。多くの場合は後に述べるケトーシスを合併している。

図 1 に，代謝性アシドーシス（pH ＜7.3，HCO_3^- ＜15 mmol/L）の鑑別フローチャートを示す。これは Saudubray らのアルゴリズムを改変したもの[1]であるが，血液ガス，血糖，アンモニア，乳酸の値に基づいている。最初にケトーシスの有無で大きく分けられている。原図ではケトーシスの「ある」・「なし」で分類されていたが，ここではあえて括弧でケトーシス「強い」・「軽い」とした。状況から予想されるよりケトーシスの程度が軽い場合は，これまでのケトーシス「あり」での検討でなく，「なし」の方向での検討が必要であるためである。低血糖をきたしている症例で，血中ケトン体が 800 μmol/L であれば，低血糖時なのにケトーシスは軽いとして「なし」の方向に進まないと脂肪酸 β 酸化系異常症にたどりつかない。

代謝性アシドーシスは呼吸性に代償されるため，多呼吸を生じる。このため代謝性アシドーシスが強ければ，患者は努力呼吸，多呼吸を呈する。図 2 はその代償のモデルである。もし呼吸性代償がおきないと，血液 pH はすぐに低下するが，呼吸性代償により pH が 7.3 を大きく切ることは少ない。代償しきれずに pH が 7.3 を切ってくるとき，生理的ではない病的な代謝状態を考えなくてはいけない。それが上述の鑑別フローチャートで，pH ＜7.3，HCO_3^- ＜15 mmol/L となっている理由である。

代謝性アシドーシスの評価では，アニオン

Fukao Toshiyuki
＊ 岐阜大学大学院連合創薬医療情報研究科/大学院医学系研究科小児病態学
〔〒501-1194　岐阜市柳戸 1-1〕
TEL 058-230-6386　FAX 058-230-6387
E-mail：toshi-gif@umin.net

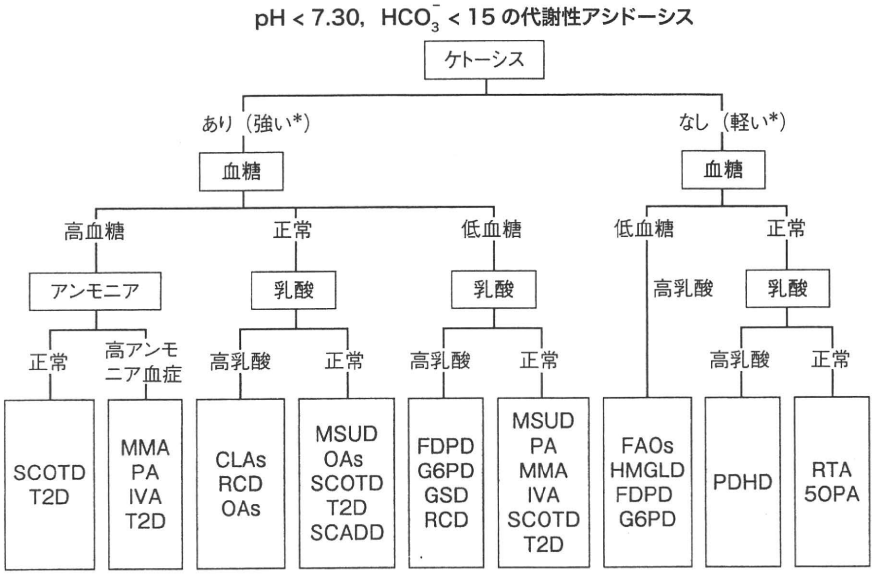


図 1　代謝性アシドーシス鑑別のフローチャート（Saudubray ら[1] 2001 の図 66-7 を改変）
SCOTD：サクシニル-CoA：3-ケト酸 CoA トランスフェラーゼ欠損症，T2D：ミトコンドリアアセトアセチル-CoA チオラーゼ欠損症，MMA：メチルマロン酸血症，PA：プロピオン酸血症，IVA：イソ吉草酸血症，CLAs：先天性高乳酸血症（ピルビン酸カルボキシラーゼ欠損症，マルチプルカルボキシラーゼ欠損症，ケトグルタル酸脱水素酵素欠損症，E3 欠損症など），RCD：呼吸鎖異常症，OAs：有機酸代謝異常症，MSUD：メープルシロップ尿症，SCADD：短鎖アシル-CoA 脱水素酵素欠損症，FDPD：フルクトースビスホスファターゼ欠損症，G6PD：グルコース 6 ホスファターゼ欠損症，GSD：グリコーゲン合成酵素欠損症，PDHD：ピルビン酸脱水素酵素欠損症，RTA：腎尿細管性アシドーシス，5OPA：5-オキソプロリン尿症　* 本文参照

A.

$$pH = 6.1 + \log\frac{HCO_3}{S \cdot PCO_2}$$

$$= 7.62 + \log\frac{HCO_3}{PCO_2}$$

B.

HCO_3 (mM)	24	15	**15**	10	**10**	5	**5**
PCO_2 (mmHg)	40	40	**28**	40	**22**	40	**16**
pH	7.40	7.19	**7.35**	7.02	**7.28**	6.72	**7.11**

図 2　代謝性アシドーシスと呼吸性代償
A：ヘンダーソン・ハッセルバルヒの式　B：血中の HCO_3^- と PCO_2 と pH の関係
太字は呼吸性代償が生じている場合を示す。

ギャップをみることも鑑別には有用である。通常，血液ガスを測定する状況では電解質も測定されているはずである。アニオンギャップ＝Na−(HCO_3＋Cl) で，正常値は 10〜14 mEq である。これが増大していることは，HCO_3^-，Cl 以外の陰性イオン（酸）が蓄積していることを意味する。

2 次，3 次スクリーニングとして，尿有機酸スクリーニングやタンデムマスによる代謝スクリーニングを行う。発作時の検体でないと診断ができない疾患や例があり，発作時の尿，血清の凍結，濾紙血採取が重要である。

II ケトーシス

一般に，アセト酢酸，3-ヒドロキシ酪酸，アセトンを総称してケトン体という。アセトンはアセト酢酸が脱炭酸して生じ，代謝的には大きな意味をもたず，呼気に排泄され，アセトン臭をきたす原因である。血液ケトン体分画は，アセト酢酸と 3-ヒドロキシ酪酸の 2 つを指し，ともにかなり強い酸であり，血液中に蓄積すればアシドーシスをきたす。

ケトン体はグルコースをセーブするための代替エネルギー源である。脂肪組織から遊離脂肪酸が切り出され，それが肝臓のミトコンドリアに取り込まれ，脂肪酸β酸化，HMG-CoAを経て，ケトン体が産生される。そのため遊離脂肪酸の動員とケトン体の動員は本来一連のことである。ケトン体産生において，①脂肪組織における遊離脂肪酸の放出，②肝臓ミトコンドリアへの脂肪酸の取り込み，③HMG-CoA合成の3ステップにおいてホルモン調節を受けており，インスリンは3ステップを強く抑え，カテコールアミン，グルカゴンは促進する。このためインスリンが優位な食後や高インスリン血症では，脂肪酸動員，ケトン体産生は抑制され，カテコールアミン，グルカゴンが優位な空腹，感染，ストレスなどの状況では脂肪酸動員，ケトン体産生は亢進する。

小児ではケトーシスを生じやすい。それは脂肪酸β酸化系-ケトン体産生系が小児における血糖維持に重要であることによる。そのため急性胃腸炎や気管支炎などで食欲が落ち，発熱などがあればすぐにケトーシスとなる。また小児では，いわゆるアセトン血性嘔吐症，ケトン血性低血糖症など名前にケトンのついた病態も存在する。これらの疾患はケトーシスをきたす頻度として多く，またケトーシス時に嘔吐，嗜眠傾向があり，発作を反復することから，臨床的に重篤感がある場合は代謝異常症の可能性も考えて十分な代謝的検討が行われるべきである。

ケトーシスの評価には尿ケトンがスクリーニングとして用いられているが，血中の遊離脂肪酸とケトン体分画を測定することが重要である。どの程度のストレス（空腹，発熱）がどの程度持続して存在した状況でのケトン体の値なのか？ 血糖と遊離脂肪酸がどの程度のときの値なのかを常に考える必要がある。24時間飢餓テスト時の遊離脂肪酸やケトン体の変化についての報告は参考になる[2]（**表**）。もちろん発熱，感染などが加われば，さらにケトン体は高くなるはずである。

ケトーシスの臨床的重要性は大きく2つに分類できる。ひとつは本来飢餓，発熱などケトーシスがあるべき状況でケトーシスがない場合である。低血糖があって，本来であればケトーシスが強いはずなのにケトーシスが軽いという低ケトン性低血糖では，脂肪酸酸化異常症，ケトン体産生障害，もしくはインスリン過分泌状態などが考えられる。ふたつめはケトーシスが強く，ケトアシドーシス（血中ケトン体7000 μmol/L以上）の場合である。この場合は異化の著しい亢進状態，有機酸代謝異常症，ケトン体利用障害などを考える必要がある[3,4]。

まれではあるが，ケトン体産生系は正常で末梢組織がケトン体を利用できないケトン体利用障害

表　空腹負荷テスト時の各種検査値の動き

	生後1か月～1歳（n=12）			1歳～7歳（n=27）			7歳～15歳（n=9）		
	15時間	20時間	24時間	15時間	20時間	24時間	15時間	20時間	24時間
血糖（mg/dl）	70.2～95.4 84.6	63.0～82.8 70.2	48.6～81.0 64.8	63.0～86.4 79.2	50.4～77.4 63.0	50.4～68.4 59.4	79.2～88.2 84.6	68.4～88.2 77.4	54～77.4 68.4
遊離脂肪酸（mmol/L）	0.5～1.6 1.0	0.6～1.3 0.9	1.1～1.6 1.3	0.6～1.5 1.1	0.9～2.6 1.7	1.1～2.8 2.1	0.2～1.1 0.7	0.6～1.3 1.0	1.0～1.8 1.4
総ケトン体（mmol/L）	0.1～1.5 0.4	0.6～3.2 1.6	1.5～3.9 2.7	0.15～2.0 0.8	1.2～3.7 2.4	2.2～5.8 3.5	<0.1～0.5 0.2	0.1～1.3 0.6	0.7～3.7 1.3
3-ヒドロキシ酪酸（mmol/L）	0.1～1.0 0.4	0.5～2.3 1.1	1.1～2.8 1.8	<0.1～0.9 0.6	0.8～2.6 1.8	1.7～3.2 2.5	<0.1～0.3 0.1	<0.1～0.8 0.4	0.5～1.3 0.9
遊離脂肪酸/総ケトン体	0.6～5.2 2.3	0.3～1.4 0.8	0.3～0.7 0.9	0.7～4.0 2.2	0.4～1.5 0.8	0.4～0.9 0.6	1.9～10.0 5.4	0.7～4.6 2.5	0.5～2.0 1.5
乳酸（mg/dl）	9.9～20.7 16.2	7.7～16.2 11.7	7.2～18.0 12.6	7.2～13.5 9.0	4.5～15.3 9.9	6.3～14.4 10.8	5.4～8.1 8.1	5.4～8.1 6.3	3.6～8.1 6.3

上段は10～90パーセンタイルを示し，下段は中央値を示す。
ケトン体は単位がmmol/Lである。7歳以降では1～7歳に比べケトン体の値が低くなっている。
空腹試験は専門医のもとで慎重に行うべきものである。
（Bonnefontら[2] 1990より一部改変）

の疾患（ミトコンドリアアセトアセチル-CoA チオラーゼ〔T2〕欠損症，サクシニル-CoA：3-ケト酸 CoA トランスフェラーゼ〔SCOT〕欠損症）がある。アセトン血性嘔吐症やケトン血性低血糖症の発作時はケトン体産生亢進によるケトーシスであり，T2 欠損症や SCOT 欠損症などのケトン体分解異常症はその産生亢進プラス利用障害によるケトアシドーシスであり，誘因などは共通である。そのため，最も重要な鑑別点はアシドーシスの程度といえる。また，アセトン血性嘔吐症やケトン血性低血糖症は 1 歳半以降の発症がほとんどであるが，ケトン体分解異常症の発症はそれ以前にピークがある。そのため，重いアセトン血性嘔吐症様の 1 歳の児をみたら基礎疾患を疑うべきである。

1）尿ケトン体

First line の臨床検査として，尿ケトン定性がある。この方法はアセト酢酸に反応するが，3-ヒドロキシ酪酸には反応しないことに注意。一般には，尿ケトンは血中ケトン体増加を反映するが，代謝評価には血中ケトン体分画の測定が重要である。尿ケトン陰性例でも血中の 3 ヒドロキシ酪酸が 1000 μmol/L をこえる場合もある。尿を凍結保存しておくことが，その後の診断に役立つ。ほとんど飲めず食べられずの胃腸炎の児は尿ケトン体が陽性であるのが普通である。低血糖でけいれんを起こした児が尿ケトン陰性であれば，疾患はしぼられる。

2）血中ケトン体分画，遊離脂肪酸

血清ではケトン体分画としてアセト酢酸と 3-ヒドロキシ酪酸の両者が測定できる。評価には遊離脂肪酸を同時測定することが必要である。

一般に記されている正常値は早朝空腹時採血で，総ケトン体 130 μmol/L 以下（アセト酢酸 55 μmol/L 以下，3-ヒドロキシ酪酸 85 μmol/L 以下）となっているが，小児では年齢や空腹時間で大きく変化する。ケトン体は食後などは 100 μmol/L 以下となるが，24 時間空腹で幼児期では 6000 μmol/L 近くまで増加する。健常者でも実に 100 倍近く増加することになる。一般に，総ケトン体が 7 mM（7000 μmol/L）以上をケトアシドーシスといい，0.2 mM（200 μmol/L）以上をケトーシスという。遊離脂肪酸/総ケトン体比もしくは遊離脂肪酸/3-ヒドロキシ酪酸比をみる（単位をともに mmol/L にそろえて比較）ことが評価に必要であり，脂肪酸酸化異常症，ケトン体産生異常症では，遊離脂肪酸は動員されてもケトン体が産生されず，遊離脂肪酸/総ケトン体比は 20 をこえる。一方，ケトン体利用障害では空腹のかなり初期から遊離脂肪酸/総ケトン体比は 0.3 以下になる。

Key Points

❶静脈血でもいいので血液ガス検査をすることが重要。一般に pH 7.30 未満，HCO_3^- 15 mmol/L 未満は代謝性アシドーシスが強いと考える。

❷代謝性アシドーシスの鑑別には，血糖，アンモニア，血中ケトン体（プラス遊離脂肪酸），乳酸が役立つ。

❸2 次，3 次スクリーニングとして尿有機酸スクリーニング，タンデムマスによる代謝スクリーニングを行うための検体保存が重要である。

❹ケトーシスの重要性は，① 本来飢餓，発熱などケトーシスがあるべき状況でケトーシスがない場合と，② ケトーシスが強く，ケトアシドーシス（血中ケトン体 7000 μmol/L 以上）の場合がある。

❺ケトン体の評価には遊離脂肪酸の同時測定が必要である。

文　献

1) Saudubray J-M, Charpentier C：Clinical phenotypes：diagnosis/algorithms. In Scriver CR, Beaudet AL, Sly WS, Valle D（eds）：Metabolic and Molecular Bases of Inherited Disease, 8th ed, McGraw-Hill, New York pp1327-1403, 2001
2) Bonnefont JP, et al：The fasting test in paediatrics：application to the diagnosis of pathological hypo- and hyperketotic states. Eur J Pediatr **150**：80-85, 1990
3) Mitchell GA, Fukao T：Inborn errors of ketone body metabolism. In Scriver CR, Beaudet AL, Sly WS, Valle D（eds）：Metabolic and Molecular Bases of Inherited Disease, 8th ed, McGraw-Hill, New York, p2327-2356, 2001
4) 深尾敏幸：ケトン体代謝異常症：特にアセトン血性嘔吐症と鑑別すべきサクシニル-CoA：3-ケト酸 CoA トランスフェラーゼ欠損症を中心に．日小児会誌 **111**：723-739，2007

まずは診断しなくては
臨床症状からの診断手順

ケトーシス

深尾敏幸

ケトーシスとは

- ケトーシスは，低血糖，高アンモニア血症，代謝性アシドーシスなどの臨床徴候と比べ，小児で高頻度にみられる臨床徴候といえる．
- 健常幼児であれば，15時間程度の空腹で容易にケトーシスをきたす．嘔吐，発熱を伴う胃腸炎などのときにはさらに容易に尿ケトン体陽性となり，ケトーシスの状態となる．これらは空腹やストレスなどへの生理的な反応としてのケトーシスであることがほとんどであるが，まれに代謝異常症が隠れている．
- 血液中に総ケトン体*1が増えた状態をいう．定義では0.2 mM（200 μmol/L*2）以上をケトーシスといい，総ケトン体が7 mM（7,000 μmol/L）以上をケトアシドーシスという．
- ケトン体は食後には0.05 mM程度となるが，24時間空腹で幼児期では6 mM近くまで増加する．実に100倍程度に増加することになる．
- ケトーシス，ケトアシドーシスに特有の臨床症状はないが，アセト酢酸が脱炭酸されたアセトンが呼気に出るため，アセトン臭が認められる．
- 血中ケトン体が直接嘔吐中枢を刺激して悪心，嘔吐をきたすというエビデンスはないが，多くの場合，原因か結果かは別として，ケトーシスやケトアシドーシスでは，悪心，嘔吐がみられ，ケトアシドーシスが強ければ，多呼吸*3がみられ，種々の程度の意識障害をきたす．
- 多くの有機酸代謝異常症などでは，一次的な有機酸の蓄積とともに血中ケトン体が上昇し，著しいアシドーシスをきたす．この場合，ケトン体も7 mM以上あれば文字どおりケトアシドーシスといえるが，それ以下でも厳密な区別は難しく，ケトアシドーシスと一般によばれている．これらの有機酸代謝異常症は鑑別のうえで重要である．

*1
アセト酢酸と3-ヒドロキシ酪酸を足したもの．

*2
日本ではケトン体の単位がμmol/Lであり，遊離脂肪酸ではmmol/Lが使われる場合が多い．

*3
代謝性アシドーシスの呼吸性代償のため．

ケトーシスを理解するために必要な知識（❶❷）

- ケトン体はグルコースをセーブするための代替エネルギー源であり，血糖の低下を防ぐために肝臓で産生される*4．
- 脂肪組織から動員された遊離脂肪酸（FFA）は肝細胞に取り込まれ，ミトコンドリアでβ酸化を受ける．脂肪酸のβ酸化系で得られる大量のアセチルCoAが基質となって，HMG-CoAを介してアセト酢酸が産生される．アセト酢酸は一部3-ヒドロキシ酪酸（3HB）に変換され，ともに血液中に放出される．これが血中ケトン体である．
- 肝外組織はこれを取り込み，3HBはミトコンドリアで再びアセト酢酸に変換される．アセト酢酸はサクシニルCoA:3-ケト酸CoAトランスフェラー

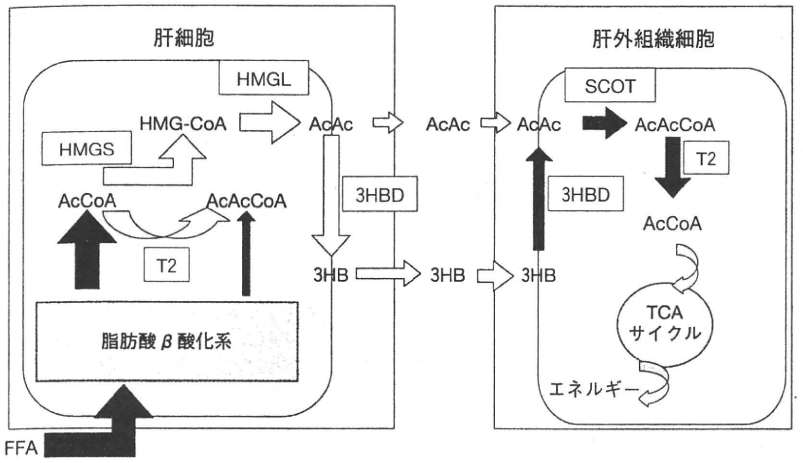
*4
ケトン体は肝臓以外のほとんどの臓器，組織が利用できるが，脳においてはとくに唯一の代替エネルギーである．

FFA：free fatty acid

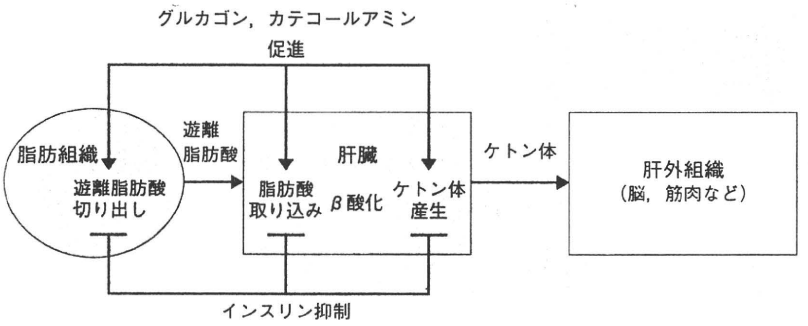
HMG-CoA：3-hydroxy-3-methylglutaryl-CoA（3-ヒドロキシ-3-メチルグルタリルCoA）

3HB：3-hydroxybutyric acid

❶ケトン体代謝



❷ケトン体代謝の調節



血糖	遊離脂肪酸	ケトン体	考えられる病態
低血糖	低い	低い	インスリン過分泌
低血糖	高い	低い	β酸化異常，ケトン体産生異常
低血糖	高い	高い	通常の低血糖（一部代謝異常症を含む）
高血糖	高い	高い	糖尿病，ストレス状態（一部代謝異常症を含む）
高血糖	低い	低い	食後

インスリンおよびグルカゴン，カテコールアミンの作用が産生を調節．

ゼ（SCOT）とミトコンドリアアセトアセチルCoAチオラーゼ（T2）によりアセチルCoAとなり，TCAサイクルに入ってエネルギーを産生することになる*5．

このように脂肪酸のエネルギーを肝臓から肝外組織に運搬するのがケトン体である．

上記のケトン体産生において，①脂肪組織における遊離脂肪酸の放出，②肝臓ミトコンドリアへの脂肪酸の取り込み，③HMG-CoA合成，の3ステップにおいてホルモン調節を受けており，インスリンは3ステップを強く抑え，カテコールアミン，グルカゴンは促進する*6．

ケトーシスの評価

ケトーシスを評価して，代謝異常症の可能性を考えるうえで，以下の点に

AcCoA：アセチルCoA

AcAcCoA：アセトアセチルCoA

AcAc：アセト酢酸

HMGS：ミトコンドリアHMG-CoAシンターゼ

HMGL：HMG-CoAリアーゼ

3HBD：3-ヒドロキシ酪酸デヒドロゲナーゼ

SCOT：succinyl-CoA:3-keto-acid CoA-transferase

T2：acetoacetyl-CoA thiolase

TCA：tricarboxylic acid

*5
この肝外組織でケトン体利用に働くSCOT欠損症，T2欠損症については3章“ケトン体利用異常症”および“β-ケトチオラーゼ欠損症”参照．

*6
このためインスリンが優位な食後や高インスリン血症ではケトン体産生は抑制され，カテコールアミン，グルカゴンが優位な空腹，感染，ストレスなどの状況下ではケトン体産生は亢進する．

注意する.

ケトーシスの程度の評価が必要

- それには尿ケトン体のみでは不十分で，血中ケトン体の測定が望ましい.

ケトアシドーシスの程度の評価には血液ガス所見が重要

- pH が 7.30 を切るアシドーシスがあるのか，代償されているのか？ 生理的なケトーシスでは pH は代償されていることが多く，病的な場合，代償しきれず pH が低いことが多い.

ケトーシスの程度の評価には血中ケトン体分画とともに血糖，遊離脂肪酸の同時測定が望ましい

- 血糖，遊離脂肪酸，ケトン体の測定は，低血糖の鑑別にも役立つ（❷）．低血糖を防ぐ反応として，遊離脂肪酸とケトン体のバランスがとれた生理的状態であるかの評価が遊離脂肪酸/総ケトン体比（もしくは遊離脂肪酸/3HB）をみることで可能となる.
- 一般には遊離脂肪酸/総ケトン体比は 1 に近いが，飢餓が進むと値は徐々に低くなる．ケトン体利用障害では空腹のかなり初期から遊離脂肪酸の動員以上に血中にケトン体が著しく増加し，遊離脂肪酸/総ケトン体比は 0.3 以下になる*7.

臨床的判断材料（たとえばどの程度のケトン体産生亢進刺激のある状態か）の情報が必要*8

- 飢餓や発熱，感染，ストレス時にケトン体は著しく産生されるので，その値が臨床的に不自然に高い，低いという判断が必要になる*9.

ケト（アシド）ーシスの鑑別（❸）

- **鑑別のための検査**：ケトン体以外に血糖，血液ガス，アンモニア，乳酸，ピルビン酸（first line 検査），および発作時の尿有機酸分析，血清や濾紙血のアシルカルニチン分析（second line 検査）が必要.
- 一般には生理的なケトーシスである空腹，異化状態，胃腸炎，アセトン血性嘔吐症，反復性ケトン性低血糖などでは強いアシドーシス（pH＜7.30，HCO_3^-＜15 mmol/L）はまれである*10．この場合は❸でみると「アシドーシスなし」に進み，血糖値，持続性ケトーシス，肝腫大の有無で鑑別する.
- 持続性ケトーシス（持続性尿ケトン体陽性）はまず SCOT 欠損症を疑う特徴的所見である.
- アシドーシスを伴う（pH＜7.30，HCO_3^-＜15 mmol/L）場合は，多くの有機酸の蓄積する疾患が鑑別にあがることになる．前述の second line 検査が必要である.

いわゆるアセトン血性嘔吐症，ケトン性低血糖症

- これらの疾患はケトーシスをきたす頻度として多く，またケトーシス時に嘔吐，嗜眠傾向があり，発作を反復することから，臨床的に重篤感がある場合は初回発作時に十分な代謝的検討を行うべきである.
- アセトン血性嘔吐症やケトン性低血糖症の発作時はケトン体産生亢進によ

*7
24 時間空腹負荷試験における血糖，遊離脂肪酸，ケトン体の変化は 1 章 "first line 検査" の "ケトン体" の❸（p.94）参照.

*8
食後 2 時間なのか，15 時間空腹なのか，頻回の嘔吐で食事摂取できない状態が 10 時間続いたとか，39°C の発熱が 2 日あるなど．の情報を得る.

*9
24 時間空腹負荷試験での値は発熱時や嘔吐時にはあてはまらない.

HCO_3^-：重炭酸イオン

*10
実際 pH＜7.30 を下回ることもある．その場合は精査.

注意すべきは T2 欠損症や SCOT 欠損症，その他の有機酸代謝異常症でもアシドーシス発作のストレス状態から血糖が，糖尿病性ケトアシドーシスほどではないものの，高値をとることがある．SCOT 欠損症や T2 欠損症では通常低血糖はきたしにくいが，新生児期や早期乳児期発症例では発作時低血糖をきたす症例もある.