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Enzymatic diagnosis of medium-chain acyl-CoA dehydrogenase deficiency by detecting 2-octenoyl-CoA production using high-performance liquid chromatography: A practical confirmatory test for tandem mass spectrometry newborn screening in Japan

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Abstract

Many of the previously described enzymatic assay methods for the diagnosis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency have been dependent upon the measurement of radioisotope-labeled co-products or reduction of electron acceptors. We have developed a direct assay method to detect 2-enoyl-CoA production using high-performance liquid chromatography (HPLC). Crude cell lysate prepared from lymphocytes were incubated with *n*-octanoyl-CoA and ferrocenium hexafluorophosphate. The detection of 2-octenoyl-CoA was significantly reproducible. We applied the assay to samples from four infants suspected to have MCAD deficiency by tandem mass spectrometry (MS/MS) newborn screening conducted in the Hiroshima area of Japan. Three of them were proved to have pathologically reduced residual enzyme activities, although they were associated with various clinical and biochemical phenotypes. In addition, another symptomatic Japanese patient and her presymptomatic sibling who were detected by MS/MS selective screening were successfully diagnosed by our enzymatic assay. These results indicate that the method can be a useful confirmatory test for MS/MS screening of MCAD deficiency.

Keywords: Medium-chain acyl-CoA dehydrogenase; Enzymatic assay; 2-Octenoyl-CoA; High-performance liquid chromatography; Tandem mass spectrometry; Newborn screening; Japanese

1. Introduction

Tandem mass spectrometry (MS/MS) has enabled screening of inborn errors of fatty acid oxidation, including medium-chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3) deficiency. In order to aid the timely follow-up of screening results that suggest abnormalities in MCAD, rapid and simple confirmatory tests for the enzyme activity and/or gene mutation analysis should be available. For the enzymatic study, the assay which uses the reduction of electron transfer flavoprotein (ETF) has been utilized as a standard method [1–3]. However, since this method requires purification of pig liver ETF and must be performed under strictly anaerobic conditions, it is disadvantageous and other artificial electron acceptors, such as ferrocenium ion [3,4], and phenazine methosulfate (PMS) in combination with dichlorophenol indophenol (DCIP) [5], have been utilized. The assay for

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tritium release from [2,3-^{3}H]acyl-CoA was described as a radioisotope-dependent method [6]. In addition, intact-cell oxidation assays using the $^{14}CO_2$ release [1,4,5] and the tritium release from [9,10-^{3}H]fatty acids [7–9] were frequently utilized in previous studies.

Despite being the main product of the enzymatic reaction in the assays mentioned above, 2-enoyl-CoA production is not directly detected in these methods. There were several reports on the product formation in such assays. Kølvraa et al. detected the production of 3-hydroxyfatty acid using gas chromatography mass spectrometry (GC/MS) by coupling 2-enoyl-CoA production with crotonation and alkaline hydrolysis [10], and this method was utilized in other studies [11,12]. Wanders [13] and Oey [14] referred to their method that detects the enoyl-CoA species produced by dehydrogenation of 3-phenylpropionyl-CoA using high-performance liquid chromatography (HPLC); however, the details of the method have not been described. In order to realize a practical method for enzymatic diagnosis of MCAD deficiency, we have developed another HPLC-based assay wherein *n*-octanoyl-CoA is used as substrate and 2-octenoyl-CoA production is directly detected. In this report, we will also demonstrate the application of our method to confirming MCAD deficiency in patients found through an MS/MS newborn screening program conducted in Japan.

2. Experimental

2.1. Reagents

n-Octanoyl-CoA (MW 893.7) and flavin adenine dinucleotide (FAD) were purchased from Sigma Chemical (St. Louis, MO, USA). Ferrocenium hexafluorophosphate ($FcPF_6$) was purchased from Aldrich (St. Louis, MO, USA). Acyl-CoA oxidase (ACO) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All the other chemicals used were of the highest purity commercially available.

2.2. Preparation of crude cell lysate

Human lymphocytes were isolated from venous blood samples. Informed consent was acquired prior to blood sampling. Heparinized blood was diluted 1:1 with saline and layered over SEPARATE-L lymphocyte isolation medium (Muto Pure Chemicals, Tokyo, Japan). After centrifugation at 25 °C for 30 min at 400 × *g*, lymphocytes were isolated and washed twice with saline, each time followed by centrifugation at 4 °C for 10 min at 200 × *g*. The number of lymphocytes obtained was counted, and the cells were washed again with saline followed by centrifugation at 4 °C for 10 min at 400 × *g*. Subsequently, the lymphocytes were transferred to other tubes and centrifuged at 4 °C for 5 min at 7200 × *g*. The saline was discarded, and the final cell pellets were either used directly or kept at −80 °C. Immediately before analysis, distilled water was added to the pellets to achieve a cell density of 10^6 lymphocytes/50 μl, and the cells were lysed by pulsed sonic disruption (1 cycle/s with 30% duty cycle of sonic burst at 45 W, using SONICATOR W-225R; Misonics, New York, USA) that took 2 min under ice bath conditions. Complete disruption of the cells was ascertained by microscopic examination. Immediately following sonication the crude cell lysate was used for the reaction.

2.3. Enzymatic reaction

n-Octanoyl-CoA and $FcPF_6$ were dissolved in distilled water just prior to each analysis. The reaction mixtures were comprised of 80 mmol/l K_2HPO_4 (pH 7.0), 2 mmol/l *n*-octanoyl-CoA, 1 mmol/l $FcPF_6$, and a 50 μl aliquot of the crude cell lysate, resulting in a total volume of 100 μl. FAD was added to some samples and omitted in some other samples. The mixture was incubated at 37 °C, and the reaction was terminated by adding 100 μl of acetonitrile. Control blanks, to which acetonitrile was added before incubation, were prepared for each assay. Denatured protein and other insoluble constituents were precipitated by centrifugation at 7200 × *g* at 4 °C for 10 min, and a 20-μl aliquot of the supernatant was analyzed by HPLC.

2.4. HPLC analysis

The product of the enzymatic reaction and the other constituents were separated by an HPLC system (LC-10AD; Shimadzu, Kyoto, Japan) equipped with a reverse-phase octadecylsilane column of 150 mm × 6.0 mm (STR ODS-II; Shinwa Chemical Industries, Kyoto, Japan). The mobile phase was composed of 100 mmol/l NaH_2PO_4 (pH 4.0) and 28% (v/v) acetonitrile, and it was pumped at a flow rate of 1.5 ml/min. The CoA-derivatives were detected at 260 nm using an ultraviolet spectrophotometric detector (SPD-6A, Shimadzu). The peak area was quantified using a data processing system (C-R4A chromatopac, Shimadzu). Lacking a commercially available authentic standard for 2-octenoyl-CoA, we performed the identification and quantification as follows.

2.5. Identification of 2-octenoyl-CoA

Since the amount of product in the assay for dehydrogenase activity toward *n*-octanoyl-CoA (hereafter MCAD activity) was limited for further analysis, we utilized the ACO activity toward *n*-octanoyl-CoA, using modifications of a previous report [15]. The product, theoretically considered to be 2-octenoyl-CoA, was separated by HPLC. The mobile phase was composed of 100 mmol/l CH_3COONH_4 (pH 4.0) and 30% (v/v) acetonitrile, with the flow rate set at 1.5 ml/min. The fraction of the product was collected and introduced into a time of flight mass spectrometer equipped with an electrospray ion source (ESI-TOFMS; Q-STAR XL;

Applied Biosystems, Foster City, CA, USA). Scan range was *m*/*z* 100–1000 in the negative ion mode.

2.6. Quantification of 2-octenoyl-CoA

The collected fraction of the product of the ACO reaction was dried at 25 °C in a centrifugal vaporizer (CVE-200D; Tokyo Rikakikai, Tokyo, Japan). The resultant material was dissolved in distilled water, and the absorbance at 258 nm was quantified using a spectrophotometer (Hitachi 557, Tokyo, Japan). The concentration of 2-octenoyl-CoA was calculated based upon a previous report that determined the extinction coefficient of 2-octenoyl-CoA to be 20.4 mM^{-1} cm^{-1} at 258 nm [16]. The same samples were re-analyzed by HPLC under the condition used for the assay of MCAD activity (hereafter MCAD assay) to determine the correlation between the peak area and the concentration of 2-octenoyl-CoA.

2.7. Identification of mutations in the MCAD gene

Genomic DNA was isolated from white blood cells by standard laboratory procedures. Polymerase chain reaction (PCR) was used to amplify all twelve exons, including part of the flanking intron sequences, of the human MCAD gene, with intron-located primers as previously described [17], under standard conditions in an automated thermal cycler (DNA Thermal Cycler 480; Perkin-Elmer, Wellesley, MA, USA). PCR products were separated on 2% agarose gel and were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA). Cycle sequencing was performed using DNA BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems).

3. MS/MS newborn screening in the Hiroshima area

We have conducted a pilot study of MS/MS newborn screening in the Hiroshima area of Japan since 1999. All newborns in the area whose parents gave written informed consent in advance were enrolled in this study. Generally, dried blood spots (DBS) were collected on the fifth day after birth, and were analyzed by MS/MS. Details of the MS/MS protocol were described elsewhere [18]. Newborns showing elevated levels of octanoylcarnitine (C8-carnitine) in DBS (cut off < 0.3 nmol/ml) associated with the ratio of octanoylcarnitine to decanoylcarnitine (C8/C10) higher than 1.5 were suspected to have MCAD deficiency.

In a period from April 1999 through October 2004, 101,020 out of 154,998 newborns (65.2%) in the Hiroshima area consented to participate in this screening. Octanoylcarnitine levels were abnormally elevated in DBS obtained from four newborns. One of them was an extremely-low-birth-weight (ELBW) infant who was supplemented with medium-chain triglyceride oil; the C8-carnitine concentration and the C8/C10 ratio in DBS were 0.62 nmol/ml and 5.17, respectively. He was proved to have normal MCAD activity afterward. Profiles of the other three suspected cases are summarized in Table 1. There was no consanguinity in any of the three families.

Table 1
Profiles of the patients diagnosed with MCAD deficiency

	Patient 1	Patient 2	Patient 3
Year of birth	2000	2001	2003
Sex	Male	Female	Male
Ethnicity	Father: Japanese–Peruvian Mother: Japanese–European	Japanese	Japanese
C8 in neonatal DBS[a] (C8/C10 ratio)[b]	0.62 (3.65)	5.92 (11.38)	0.43 (1.87)
Highest C8 in serum[c] (C8/C10 ratio)[b]	5.97 (3.49)	19.56 (11.93)	1.21 (2.24)
Hexanoylglycine in urine	Elevated (in acute metabolic failure)	Elevated (in neonatal period)	Not elevated
Hypoketotic dicarboxic aciduria	Detected (in acute metabolic failure)	Detected (in neonatal period)	Not detected
MCAD activity[d]	10.3%	2.6%	13.2%
MCAD gene mutation (paternal/maternal allele)	449–452delCTGA/157C > T	Unknown/unknown	Unknown/449–452delCTGA
Clinical presentation	Acute metabolic failure (cardiopulmonary arrest) at 8 months old	Asymptomatic as of 42 months old	Asymptomatic as of 16 months old

[a] Concentration of octanoylcarnitine in dried blood spots for newborn screening, expressed as nmol/ml (cut off < 0.3).
[b] The ratio of octanoylcarnitine to decanoylcarnitine in dried blood spots or in serum. The ratio higher than 1.5 associated with the elevation of octanoylcarnitine is indicative of MCAD deficiency.
[c] Concentration of octanoylcarnitine in serum, expressed as nmol/ml (cut off < 0.2).
[d] *n*-Octanoyl-CoA dehydrogenase activity, expressed as percentage of the mean of those in samples from 15 normal subjects.

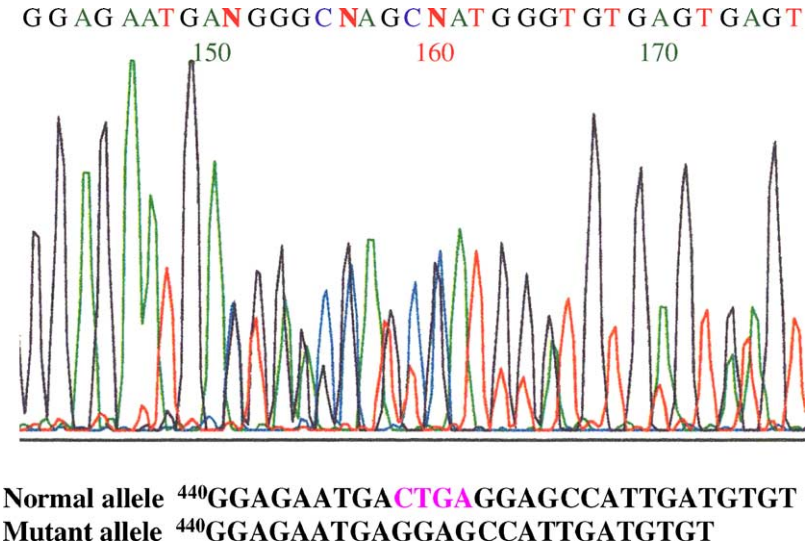

Fig. 1. A part of the sequence of exon 6 in a sample collected from patient 3. Direct sequencing identified a heterozygous deletion of four base pairs (CTGA) at the sites 449–452.

4. Case reports

Patient 1 was a healthy 8-month-old boy of Japanese–Peruvian–European descent born in 2000, who abruptly became ill and rapidly fell into cardiopulmonary arrest associated with liver dysfunction and hyperammonemia (AST 286 IU/l, ALT 257 IU/l, CK 8702 IU/l, $NH_3 > 400$ μg/dl). Plasma glucose at the onset was not measured. Fortunately, resuscitation and intensive care were started early, so that he recovered without any sequelae. The concentration of C8-carnitine and the C8/C10 ratio in serum collected during the acute symptomatic period were 5.97 nmol/ml (cut off < 0.2) and 3.49, respectively, and those in DBS for newborn screening were 0.62 nmol/ml and 3.65, respectively. The organic acid profile of urine was analyzed by GC/MS according to a previously reported method [19]; hypoketotic dicarboxylic aciduria and elevated hexanoylglycine were evident. The concentration of C8-carnitine in serum collected during the period of recovery from the metabolic failure decreased to a level slightly above the cut off value (0.41 nmol/ml). We did not follow this patient any longer because he moved away from the Hiroshima area soon after the recovery.

Patient 2 was a Japanese girl born in 2001 as a mature infant without any familial history suggestive of metabolic disorders. The concentration of C8-carnitine in DBS was 5.92 nmol/ml, with the C8/C10 ratio being 11.38. When she came to our clinic on the 25th day after birth for further examination, there was no abnormal finding in her clinical presentation, blood gas analysis, or in the levels of blood glucose, plasma ammonia, and serous transaminases. Analysis of urine collected on her first visit by GC/MS revealed non-ketotic dicarboxylic aciduria and elevation of hexanoylglycine. She has been asymptomatic until 42 months, though the profiles of acylcarnitines in serum have always been highly abnormal; the concentrations of C8-carnitine reached 19.56 nmol/ml at highest, when the C8/C10 ratio was 11.93.

Patient 3 was a Japanese boy born in 2003. There was no particular problem in his familial, fetal, or perinatal history. The concentration of C8-carnitine in DBS was 0.43 nmol/ml, and the C8/C10 ratio was 1.87. Organic acid profile of urine collected at the age of 1 month was normal. He has been asymptomatic until 16 months old, with the concentration of C8-carnitine always slightly above the cut off value (1.21 nmol/ml at highest with the C8/C10 ratio being 2.24).

Two different mutations in the MCAD gene were identified in the samples collected from the three patients; a novel mutation of 449–452delCTGA (exon 6) in one allele of patients 1 and 3 each (Fig. 1), and a single base transition of 157C > T (exon 3) in the other allele of patient 1. Regarding patient 1, the del449–452 mutation derived from his father of Japanese–Peruvian descent, and the 157C > T mutation from his mother of Japanese–European descent. Patient 3 inherited the allele harboring the four-base deletion from his mother. No mutation was identified in the other allele of patient 3, or in either allele of patient 2.

5. Results

Kinetic studies on the MCAD activity were initially performed using crude cell lysate prepared from 10^6 lymphocytes. Product formation linearly increased within the range of *n*-octanoyl-CoA concentration from 0.5 to 4 mmol/l ($n = 4$, Fig. 2a). Product formation with regard to $FcPF_6$ concentration and incubation time exhibited an increase with a tendency to reach a plateau within the range tested; 0.25–4 mmol/l of $FcPF_6$ ($n = 3$, Fig. 2b), and 5–30 min of incubation ($n = 3$, Fig. 2c). Adding FAD to the reaction mixture at concentrations of 0.1, 1, and 10 mmol/l did not

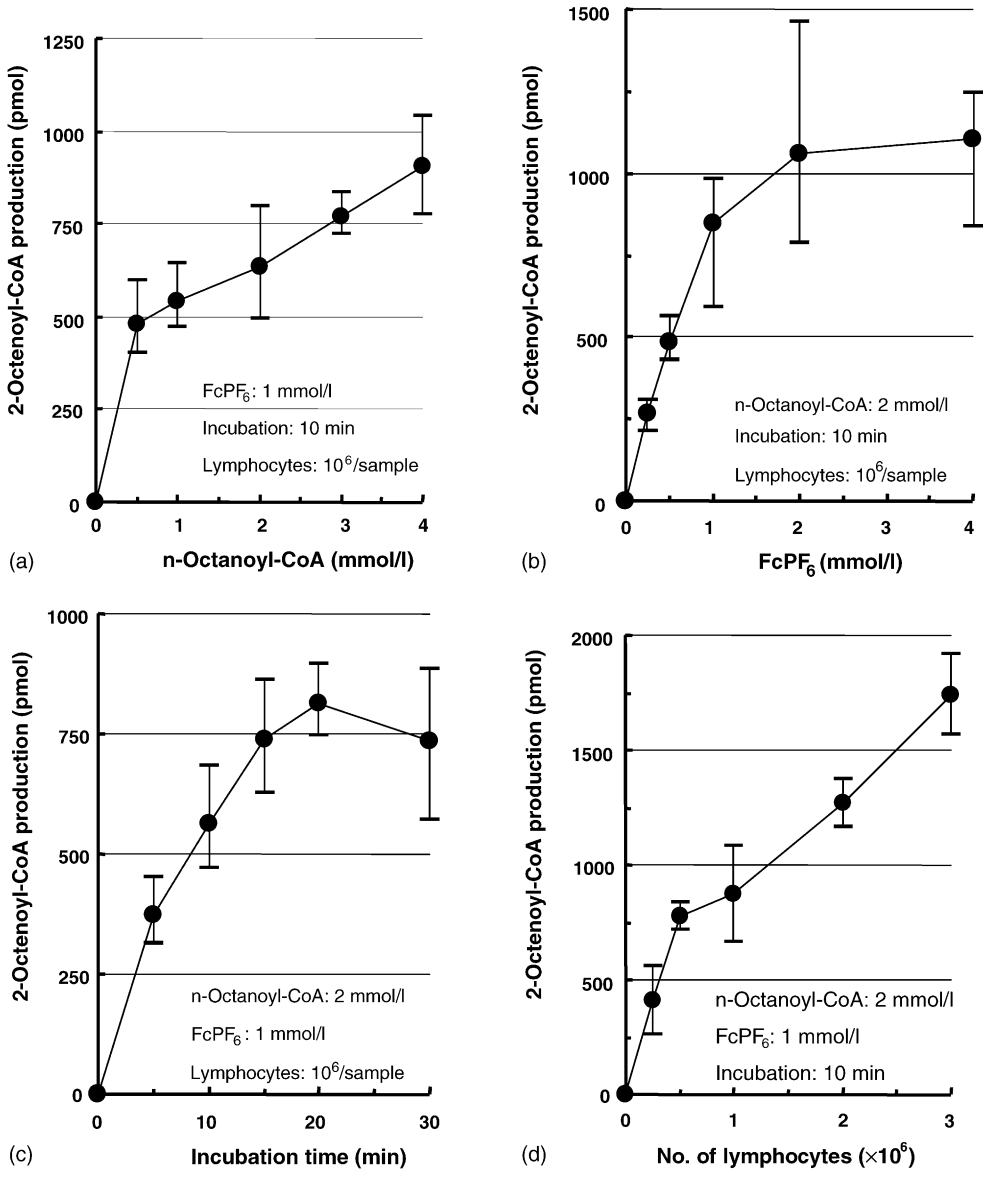

Fig. 2. Rate of 2-octenoyl-CoA production by crude cell lysates prepared from lymphocytes of normal subjects, as a function of (a) *n*-octanoyl-CoA concentration ($n = 4$); (b) $FcPF_6$ concentration ($n = 3$); (c) incubation time ($n = 3$); and (d) density of lymphocytes in the crude cell lysate ($n = 2$). Each point of the data represents the mean and the range of the values.

affect product formation (data not shown). When the concentrations of *n*-octanoyl-CoA and $FcPF_6$ in the reaction mixture were kept at 2 mmol/l and 1 mmol/l, respectively, and the incubation time was kept 10 min, product formation increased linearly depending on the initial cell density in the crude cell lysate. This cell density was within the range of 0.25×10^6–3×10^6 lymphocytes/50 μl ($n = 2$, Fig. 2d). Based on these results, we determined the assay condition as follows; the reaction mixture should contain the crude cell lysate prepared from 10^6 lymphocytes, 2 mmol/l *n*-octanoyl-CoA, and 1 mmol/l $FcPF_6$, with the incubation for 10 min.

Fig. 3a shows a representative chromatogram of the MCAD assay in a sample from a normal subject. The peak of the product that was assumed to be 2-octenoyl-CoA was eluted after an 8-min retention time and was well resolved from the other constituents. The retention time was compatible to that of the product of the ACO reaction. Analyzed by ESI-TOFMS, this compound presented a base peak of *m*/*z* 444.5, while the mass spectrum of the base peak for authentic *n*-octanoyl-CoA was *m*/*z* 445.5, both corresponding to doubly-deprotonated molecular ions ($[M - 2H]^{2-}$; Fig. 3b and c). These results demonstrated that the peak obtained in the chromatogram of the MCAD assay was indicative of 2-octenoyl-CoA. The concentration of 2-octenoyl-CoA dissolved in distilled water that was calculated from the absorbance at 258 nm correlated linearly with its peak area on HPLC within the range of 1.03–102.94 pmol per 20 μl of samples introduced into HPLC (Fig. 4). Based on the method from IUPAC provisional draft (Currie LA, 1994), detection limit and quantification limit of 2-octenoyl-CoA

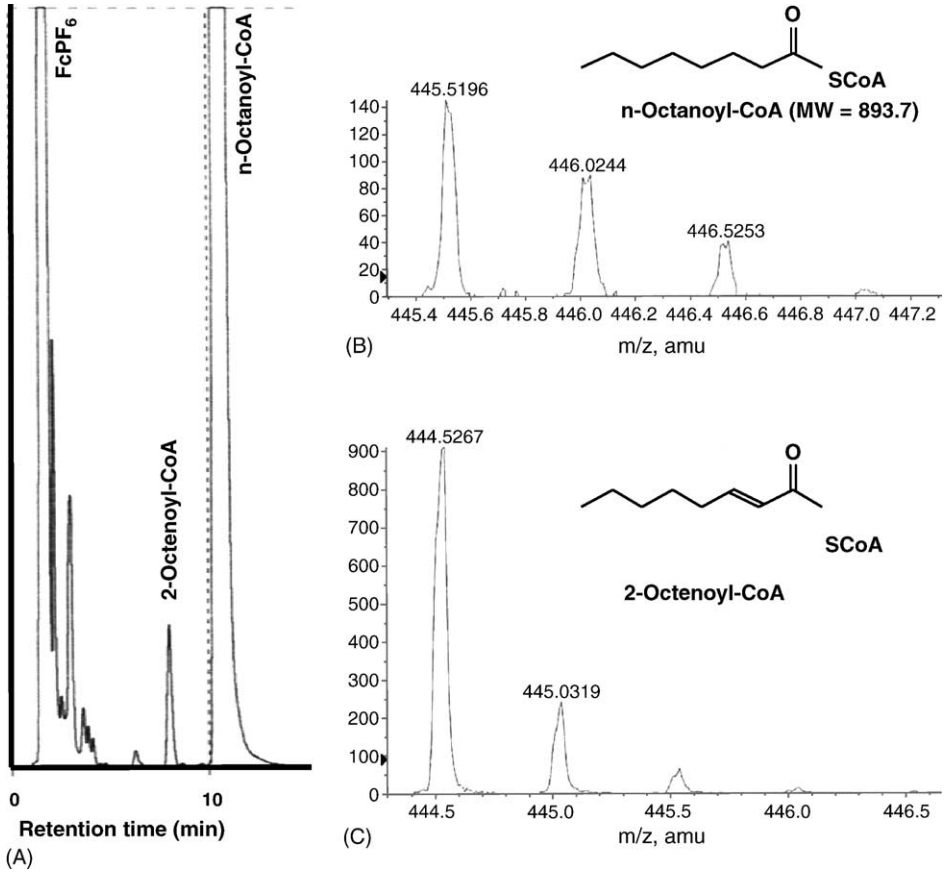

Fig. 3. (a) A representative chromatogram of the assay for the MCAD activity in lymphocytes from a normal subject. The product of the reaction was eluted at a retention time of approximately 8 min. The retention time was compatible to that of the product of the ACO reaction. Analyzed by ESI-TOFMS, (b) authentic *n*-octanoyl-CoA presented a base peak of *m*/*z* 445.5, while (c) the fraction of the product of the ACO reaction contained a compound presenting a base peak of *m*/*z* 444.5. Both mass spectra corresponded to $[M-2H]^{2-}$ molecular ions.

were determined to be 0.62 and 1.87 pmol, respectively. The detection limit was lower than approximately 2% of the mean 2-octenoyl-CoA production in samples from normal subjects. In the above conditions, the MCAD activity was determined as a mean of duplicated assays with subtraction of a blank value, expressed as pmol 2-octenoyl-CoA/min per 10^6 lymphocytes. Thus, activity that is as low as 0 pmol/min per 10^6 lymphocytes can theoretically be determined. Coefficient of variation of the intra-assay ($n=5$) was 6.6% and that of the inter-assay conducted on five consecutive days ($n=5$) was 4.0%.

The MCAD activities in the samples collected from normal subjects ($n=15$) ranged from 24.96 to 40.92 pmol/min per 10^6 lymphocytes, with a mean ± S.D. value of 32.96 ± 5.47 pmol/min per 10^6 lymphocytes, whereas those in the samples collected from patients 1, 2, and 3 were 3.40, 0.85, and 4.34 pmol/min per 10^6 lymphocytes, respectively. Residual activities of the three patients were calculated to be 10.3%, 2.6%, and 13.2% of the mean of those in normal subjects, respectively (Fig. 5). The MCAD activity in the lymphocytes of the ELBW infant, who showed abnormal results in the MS/MS screening, was normal (107.4% of the mean of those in normal subjects).

In addition, we applied the enzymatic assay to another symptomatic Japanese girl, who was the first confirmed patient of MCAD deficiency in Japan and was referred to in our previous report [18] (patient 4), and her younger brother who was presymptomatically found to have elevated levels of C8-carnitine (patient 5). The MCAD activities in their lymphocytes were 3.2% and 2.4% of the mean of those in normal subjects, respectively. More detailed information about these siblings will be described elsewhere (Ohtake et al., in preparation).

The MCAD activity of the mother of patient 3, who had the same deletion in one allele of the MCAD gene, was 12.97 pmol/min per 10^6 lymphocytes (39.4% of the mean of those in normal subjects), while that of his father was slightly below the range of the normal control value (23.18 pmol/min per 10^6 lymphocytes, or 71.9% of the mean of those in normal subjects). Comparatively, the enzyme activities in lymphocytes of the mother of patient 2, and the father and the mother of patients 4 and 5, were 32.2%, 36.5%, and 27.0% of the mean of those in normal subjects, respectively. Distribution of the MCAD activities of these carriers is also presented in Fig. 5.

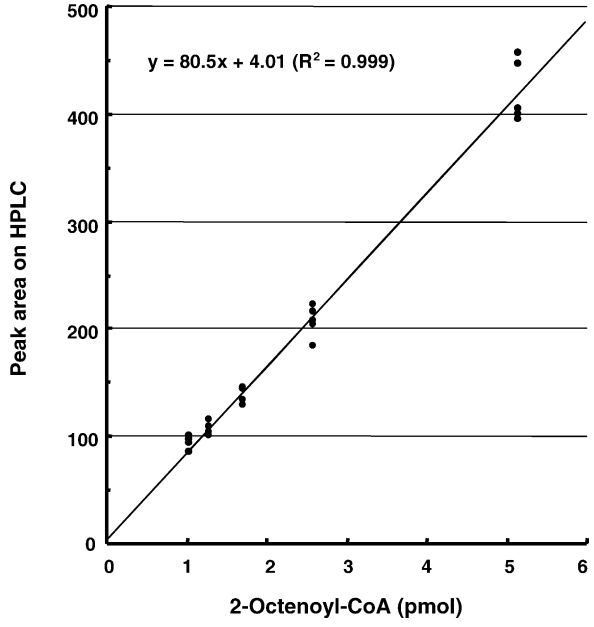

Fig. 4. A regression line between the amount of 2-octenoyl-CoA and the peak area on HPLC is described within the lower part of the range studied. 2-Octenoyl-CoA dissolved in distilled water was introduced into the HPLC at eight different concentrations ranging from 1.03 to 102.94 pmol per 20 μl of sample and repeated five times for each concentration.

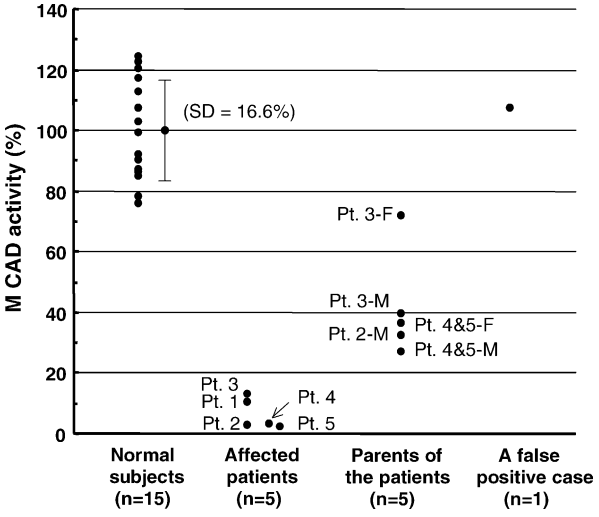

Fig. 5. The MCAD activities in samples from normal subjects ($n = 15$), patients with MCAD deficiency ($n = 5$), their parents ($n = 5$), and a false positive case, measured under the determined assay condition, are shown as percentage of the mean of those in normal subjects. Residual MCAD activities of the patients ranged from 2.4% to 13.2%. The enzymatic activities of their parents ranged between those of the patients and the normal subjects (27.0–71.9%). The very-low-birth-weight neonate supplemented with medium-chain triglyceride oil had normal level of MCAD activity (107.4%).

6. Discussion

The introduction of acylcarnitine analysis by MS/MS has enabled mass screening of fatty acid oxidation disorders including MCAD deficiency. As the application of this technique to newborn screening prevails, there will be increasing need for simpler tests to confirm the abnormal results indicative of MCAD deficiency, especially in cases that do not have the common 985A > G mutation in the MCAD gene. Thus, it is worth establishing a rapid and simple method of enzymatic diagnosis. Various methods have been described in previous reports and are summarized in Table 2. In a previous review [13], Wanders et al. pointed out the following demerits of these conventional methods: use of radioisotopes ($^{14}CO_2$ release, tritium release), requirement of commercially unavailable reagents (tritium release from [2,3-^{3}H]acyl-CoA, ETF reduction), demand of anaerobic conditions (ETF reduction), high background activity ($FcPF_6$ reduction, PMS/DCIP reduction), or need for derivatization (GC/MS-based assay). They suggested that their HPLC-based product formation assay was superior, and using this method Oey et al. reported some data on the activities of MCAD and other fatty acid oxidation enzymes in human placenta [14]. However, based on the information referred by us, neither details of their method, nor data obtained by applying the method to patients with MCAD deficiency have been described.

We also intended to develop an MCAD assay method for directly detecting 2-enoyl-CoA formation. Since it was previously reported that lymphocytes could be used for enzymatic assay of MCAD [1,4,13], we have shortened the assay process by using peripheral lymphocytes as a crude enzyme source. Although the dehydrogenase activity toward *n*-octanoyl-CoA in a crude cell lysate can be influenced by the similar enzymes located in peroxisomes [20], Wanders et al. showed that such peroxisomal contribution should be only minimal [4]. The analytical procedure was simplified by adopting HPLC, abolishing the need of radioisotopes and further derivatization of the product. Thus, it only takes several hours from sampling of blood to the end of the assay. The amount of the blood required of newborns is a few milliliters of whole blood, which is usually adequate to isolate sufficient number of lymphocytes that are required for repeated assays. Concerning the substrate, Wanders et al. used 3-phenylpropionyl-CoA [13], which was shown to be highly specific to MCAD, least influenced by overlapping chain length specificities of other acyl-CoA dehydrogenases [21]. However, the superiority of this compound diminishes, because it has to be synthesized in each laboratory. For practical purpose, it is desirable to establish a reliable assay method by using *n*-octanoyl-CoA, which has been utilized in many of the previous studies (Table 2), and is commercially supplied.

The levels of residual MCAD activity in our three patients seem to be either consistent with or superior to those in previous reports (Table 2). Patient 1 abruptly developed severe metabolic decompensation associated with elevated levels of medium-chain acylcarnitines in serum, and the diagnosis of MCAD deficiency was confirmed both enzymatically and genetically. Compared with this case, patient 2 had always shown severer biochemical abnormalities, and the residual MCAD activity in her lymphocytes was also clearly lower

Table 2
Methods for enzymatic diagnosis of MCAD deficiency described in previous reports

Author	Method	Source of enzyme (crude cell lysate)	Substrate	Number of patients	Mean residual activity (%)
Coates [1]	ETF reduction	Fibroblasts	Octanoyl-CoA	10	9.2
		Lymphocytes		6	9.0
Frerman and Goodman [2]	ETF reduction	Fibroblasts	Octanoyl-CoA	2	(1) 9.2
					(2) <3.3
Lehman [3]	ETF reduction	Fibroblasts	Octanoyl-CoA	5	7.6
Lehman [3]	$FcPF_6$ reduction	Fibroblasts	Octanoyl-CoA	5	4.5
Wanders [4]	$FcPF_6$ reduction	Fibroblasts	Octanoyl-CoA	4	9.8
Rhead [5]	PMS/DCIP reduction	Mitochondria of fibroblasts	Octanoyl-CoA	3	5.2
Amendt [6]	Tritium release	Fibroblasts	[2,3-^{3}H]Octanoyl-CoA	19	7.8
Kølvraa [10]	3-OH-Fatty acid formation (GC/MS)	Fibroblasts	Octanoyl-CoA	1	25.0
Niezen-Koning [11]	3-OH-Fatty acid formation (GC/MS)	Fibroblasts	Hexanoyl-CoA	2	35.7
Duran [12]	3-OH-Fatty acid formation (GC/MS)	Lymphocytes	Octanoyl-CoA	5	2.3–18.8
Wanders [13]	2-Enoyl-CoA formation (HPLC)	Fibroblasts	3-Phenylpropionyl-CoA	ND	ND
		Lymphocytes			
Tajima	2-Enoyl-CoA formation (HPLC)	Lymphocytes	Octanoyl-CoA	5	6.3 (2.4–13.2)

ND: not described.

than that of patient 1. Nevertheless, no mutation was detected within the entire coding region of the MCAD gene, including exon–intron junctions. On the other hand, profiles of acylcarnitines in DBS and serum from patient 3 have been mild, and only one mutant allele has been clarified; these observations suggest that this case could be a heterozygous carrier. However, the concentrations of C8-carnitine in DBS and serum of patient 1 during non-symptomatic periods were as low as those of patient 3, and the residual MCAD activity of patient 3 was also at approximately the same level as that of patient 1. In addition, the MCAD activity of his mother, who was shown to have the same deletion in one allele, was compatible with the carrier status and was definitely higher than that of patient 3. Similar levels of residual activity were also observed in the mother of patient 2 and the parents of patients 4 and 5. These results indicate that patient 3 should also be a true patient with MCAD deficiency. Taking it into consideration that the father of patient 3 had much higher MCAD activity than the other carriers, patient 3 is supposed to have another mutation in paternal allele of the MCAD gene that should disturb the enzymatic function in a very mild fashion. According to a review by Chace et al. [22], there are cases of newborns with less than 1 nmol/ml of C8-carnitine who have become metabolically decompensated. Therefore, the severity of MCAD deficiency should not be predicted solely on the basis of C8-carnitine concentration.

Genetic heterogeneity observed in our patients suggests that it may be difficult to utilize a common mutation-specific assay as a confirmatory test for MCAD deficiency among Japanese. Although at least one allele was found to harbor the 985A > G mutation in more than 90% of Caucasian patients [23,24], previous studies on the prevalence of this mutation failed to identify any mutant allele among Japanese newborns [24–26]. To our knowledge other than 985A > G, 49 sporadic mutations have been reported [17,27–39], only two of which were definitely documented in non-Caucasian subjects [37,39]. None of these various mutations was observed in the three patients in this study, except 157C > T in one allele of patient 1, which had been identified in two symptomatic European patients [28]. Instead, 449–452delCTGA, which provokes premature stop codon at the sites 479–481, is the first pathological mutation documented in Japanese patients. Though there were cases where no mutation could be detected [29], the remaining genetic abnormalities in the patients in this study are under further investigation.

These facts and speculations highlight the importance of enzymatic diagnosis of MCAD deficiency, especially among non-Caucasian populations, and indicate that our MCAD assay method can diagnose patients with mild deficiency as well as those with severe clinical and/or biochemical phenotypes. By using this method as a confirmatory test in the MS/MS newborn screening program, we have revealed unexpectedly high frequency of MCAD deficiency in the Hiroshima area. It is estimated to range from 1:51,666 to 1:33,673, which may not be so much less than that in the Western countries; recent reports demonstrated that the frequencies of MCAD deficiency in the United States, Germany, and Australia were 1:15,000 [29], 1:20,800 [40], and 1:21,300 [41], respectively. In conclusion, our practical enzymatic diagnosis method can be a useful confirmatory test for MCAD deficiency found through MS/MS newborn screening.

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