(BSTFA + 1%TMCS) for TMS derivatization were obtained from Wako Pure Chemical Industries Ltd. The solution of *n*-alkanes (C_9–C_{33}) was obtained from Restek Corp. (Bellefonte, PA, USA).

Internal standard solution of margaric acid (MGA) and tetracosane (C_{24}) were prepared in ethyl acetate (0.5 mg/ml).

2.2. Sample preparation

The method reported by Kimura et al. [7,16] was used as the conventional method. The modified solvent extraction and TMS derivatization were described in Section 2.2.1 and Section 2.2.2, respectively.

2.2.1. Modified solvent extraction

The following preparation was performed in a 1.5 ml-glass vial (Shimadzu GLC Ltd., Tokyo, Japan). An aliquot of urine equivalent to 0.02 mg of creatinine (volume of urine is generally between 0.01 and 0.20 ml) was diluted with distilled water to adjust the final volume to 0.20 ml. To the diluted urine was then added 2 units of urease, and the mixture was incubated for 30 min at 37 °C. Four microliters of the internal standard solution (0.5 mg/ml) was then added. The mixture was acidified with 35 μl of HCl (6N) and shaken for approximately 30 s with a vortex mixer. The organic acids were then extracted with 1.2 ml of ethyl acetate by mixing vigorously for approximately 30 s with a vortex mixer. The mixture was centrifuged at 2010 × *g* for 5 min and the organic layer was treated with anhydrous sodium sulfate (0.5 g) prior to GC/MS analysis.

2.2.2. Flash-heater derivatization

The Flash-heater derivatization used in the new method was carried out as follows. A 1 μl aliquot of the extract (ethyl acetate) was sandwiched between two plugs of the BSTFA + 1%TMCS in a 10 μl injection syringe by drawing in sequence: 1 μl of BSTFA + 1%TMCS, 0.5 μl of air, 1 μl of the extract, 0.5 μl of air and 0.5 μl of BSTFA + 1%TMCS. The sample was then injected into the split/splitless injection port heated at 280 °C.

2.3. Gas chromatograph–mass spectrometer analysis

2.3.1. Gas chromatograph–mass spectrometer

The analysis were 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), a split/splitless injection port with a glass liner packed with glass wool (Restek) and a DB-5 capillary column (30 m × 0.25 mm i.d., 1.00 μm, J & W Scientific, Folsom, CA, USA). The analytical conditions of both methods are shown in Table 1.

2.3.2. Data processing

The data was processed using GCMSsolution software and GC/MS metabolite mass spectral database, which includes mass spectra and retention index data; the software and database are from Shimadzu. The retention times of target compounds were calculated from measured retention times of *n*-alkanes (C_{10}–C_{26}) and the retention indices of targets compounds stored in the database. The target compounds in the chromatogram were identified by the calculated retention times and their mass spectra [7,16,17].

2.4. Urine sample analysis

2.4.1. Analysis of urine sample spiked with organic acids

A normal urine sample was spiked with 14 organic acids (Table 2) at 50 μg/ml and internal standard solution at 10 μg/ml, and analyzed by both conventional and new methods. The retention indices of the TMS derivatives of the organic acids were determined using the measured retention times of *n*-alkanes [18].

Table 1
Analytical conditions of the conventional and new methods.

	Conventional GC/MS method	New GC/MS method
Extraction	Conventional liquid extraction	Modified liquid extraction
Derivatization	Oximation, TMS	TMS
Column	DB-5 30 m × 0.25 mm i.d. df = 1.00 μm	DB-5 30 m × 0.25 mm i.d. df = 1.00 μm
Injection method	Conventional injection (1 μl)	Flash injection (1 μl BSTFA + 1%TMCS, 1 μl sample, 0.5 μl BSTFA + 1%TMCS)
Injection temp.	280 °C	280 °C
Split mode	Split mode 1:10	Splitless mode (1 min)
Carrier gas	39 cm/s (constant velocity)	39 cm/s (constant velocity)
Column temp.	100 °C (4 min)–4 °C/min–280 °C (11 min)	100 °C (4 min)–4 °C/min–280 °C (11 min)
Interface temp.	280 °C	280 °C
Ion source	200 °C	200 °C
Data acquisition rate	0.3 s	0.3 s
Data acquisition mode	Scan	SIM

2.4.2. Analysis of normal control sample and determination of cut-off values

Urine samples were collected from 40 normal controls and analyzed by the new method. TMS derivatives of methylmalonic acid, propionylglycine, glutaric acid, isovaleryglycine, 3-methylcrotonylglycine, 3-hydroxyglutaric acid, 2-hydroxyglutaric acid, methylcitric acid were quantified as the diagnostic markers of methylmalonic acidemia (MMA), propionic acidemia (PPA), isovaleric acidemia (IVA), glutaric aciduria type I (GAI) and multiple carboxylase deficiency (MCD). The quantitative results were expressed in the form of the peak area ratios of the target compounds to the internal standard (MGA), and the mean values and the standard deviations were calculated. The cut-off value was defined as the mean plus three standard deviations.

2.5. Chemical diagnosis using the new method

Urine samples were obtained from 20 patients with the following disorders (diagnosed by the conventional method and clinical symptoms): MMA, PPA, IVA, GAI and MCD. To validate the new method each patient sample was analyzed five times and then the solvent of BSTFA + 1%TMCS as the blank sample was analyzed three times to confirm the carry over levels of target compounds.

The derivatives of the diagnostic markers with abnormal values were determined by comparing the values to the cut-off values, and their combinations allowed the chemical diagnosis [7,16,17].

3. Results

3.1. Analysis of urine sample spiked with organic acids

Table 2 shows the retention indices and the peak area ratio of the trimethylsilyl (TMS) derivatives to C_{24} (which is not derivatized) for all the TMS derivatives which were detected after five analyses of a urine sample spiked with the organic acids using the conventional and new methods.

Derivatives with different numbers of TMS-groups were formed and the main product differed between the two methods. By using the conventional method, 18 TMS derivatives were detected

Table 2
Within-run repeatability and ratio of relative recovery for organic acids added to urine.

No.	Compound[a]	m/z		Conventional method (n = 5)				New method (n = 5)				Ratio[b]
		Q-ion[c]	C-ion[c]	R.I.[d]	Peak area ratio			R.I.[d]	Peak area ratio			
					Mean[e]	SD[e]	%RSD[e]		Mean[f]	SD[f]	%RSD[f]	
1	3-Hydroxyisovaleric acid-TMS*	117	175	1032	2.440	0.570	23.4	1036	35.445	2.640	7.4	14.53
2	3-Hydroxyisovaleric acid-diTMS*	247	205	1213	4.359	0.243	5.6	1214	0.136	0.017	12.2	0.03
3	2-Hydroxyisovaleric acid-TMS*	175	157	1047	0.005	0.006	112.9	1051	0.179	0.031	17.4	35.88
4	2-Hydroxyisovaleric acid-diTMS*	219	247	1169	5.270	0.199	3.8	1171	4.605	0.268	5.8	0.87
5	Lactic acid-diTMS	219	191	1060	1.140	0.124	10.8	1062	2.991	0.324	10.8	2.62
6	3-Hydroxypropionic acid-diTMS	177	219	1144	3.731	0.236	6.3	1146	3.390	0.212	6.3	0.91
7	3-Hydroxybutyric acid-diTMS	191	233	1161	4.567	0.232	5.1	1163	1.503	0.126	8.4	0.33
8	Methylmalonic acid-diTMS	247	218	1218	4.210	0.158	3.8	1218	3.015	0.286	9.5	0.72
9	Ethylmalonic acid-diTMS	261	217	1284	5.182	0.155	3.0	1284	3.555	0.295	8.3	0.69
10	Phenyllactic acid-TMS*	205	194	1399	0.008	0.004	58.7	1399	0.029	0.045	153.4	3.83
11	Phenyllactic acid-diTMS*	194	267	1517	10.123	0.262	2.6	1517	6.966	0.381	5.5	0.69
12	Glutaric acid-diTMS	261	158	1405	11.372	0.315	2.8	1404	7.471	0.384	5.1	0.66
13	Isovalerylglycine-TMS*	216	189	1488	1.075	0.075	7.0	1487	2.965	0.185	6.2	2.76
14	Isovalerylglycine-diTMS*	288	176	1519	7.936	0.118	1.5	–	0.000	0.000	–	0.00
15	Adipic acid-diTMS	275	111	1508	6.609	0.177	2.7	1507	3.592	0.169	4.7	0.54
16	Suberic acid-diTMS	303	187	1702	7.573	0.193	2.5	1700	3.552	0.203	5.7	0.47
17	Sebacic acid-diTMS	331	215	1897	17.164	0.346	2.0	1896	6.926	0.278	4.0	0.40
18	4-Hydroxyphenyllactic acid-triTMS	308	293	1918	26.045	0.465	1.8	1917	7.023	0.313	4.5	0.27
19	Margaric acid-TMS	327	145	2145	1.918	0.036	1.9	2146	1.356	0.102	7.5	0.71
20	Tetracosane	99	67	2400	1.000	0.000	0.0	2400	1.000	0.000	0.0	1.00

[a] An asterisk indicates that two TMS derivatives were formed form an organic acid.
[b] Ratio means the relative recovery and based on ratio of the peak area ratio in the new and conventional methods.
[c] Q- and C-ions were used for selected ion monitoring (SIM).
[d] R.I. = retention index.
[e] Mean, SD and %RSD were calculated based on the peak area ratio (TMS derivatives to C24) after five analyses by the conventional method.
[f] Mean, SD and %RSD were calculated based on the peak area ratio (TMS derivatives to C24) after five analyses by the new method.

from 14 organic acids: two TMS derivatives were obtained from 3-hydroxyisovaleric acid (-TMS, -diTMS), 2-hydroxyisovaleric acid (-TMS, -diTMS), phenyllactic acid (-TMS, -diTMS) and isovaleryglycine (-TMS, -diTMS). The %RSD of the peak area ratios (TMS derivatives to C_{24}) ranged from 1.5 to 10.8% except 3-hydroxyisovaleric acid-TMS (23.4%), 2-hydroxyisovaleric acid-TMS (112.9%), phenyllactic acid-TMS (58.7%). On the other hand, by the new method 17 TMS derivatives were detected from 14 organic acids: two TMS derivatives were obtained from 3-hydroxyisovaleric acid (-TMS, -diTMS), 2-hydroxyisovaleric acid (-TMS, -diTMS) and phenyllactic acid (-TMS, -diTMS). The within-run repeatability (%RSD of the peak area ratios) ranged from 4.0 to 12.2% except 2-hydroxyisovaleric acid-TMS (17.4%) and phenyllactic acid-TMS (153.4%). Additionally the analysis was carried out every 2 days over a 10 days period and the inter-run reproducibility (%RSD of the peak area ratios) ranged from 3.4 to 13.1% for the main products.

The differences in retention indices between the two methods ranged from −2 to 4 for the same TMS derivatives.

In order to compare the recovery of the added organic acids between the two methods, the peak area ratios (organic acid to C_{24}) obtained from the new method was divided by those from the conventional method (Table 2). Those values showed more than 1.2 for 5 compounds, 0.80–1.20 for 2 compounds and less than 0.80 for 11 compounds. Additionally the recovery in percentage of the spiked value ranged from 24.6% to 115.0%.

3.2. Analysis of normal control sample and determination of cut-off values

Table 3 shows the quantitative results of the normal urine analysis and the cut-off values which were determined by the mean plus three standard deviations of the relative peak area (target compound to MGA). If the target compounds were not detected in the urine sample, the cut-off value was treated as 0.5% (the quantitative limit of MGA).

3.3. Chemical diagnosis using the new method

Table 4 shows the quantitative results for urine samples of individual patients with MMA, PPA, GAI, IVA and MCD obtained by the new method, each analyzed in five replicates. Twelve target compounds (TMS derivatives of the diagnostic markers) were detected and the %RSD ranged from 4.3% to 12.0%. Propionylglycine-diTMS, isovalerylglycine-diTMS and 3-methylcrotonylglycine-diTMS (for PPA, IVA and MCD, respectively) were not detected. However, propionylglycine-TMS, isovalerylglycine-TMS, and 3-methylcrotonylglycine-TMS were detected, and the %RSDs were 4.3%, 5.1% and 11.6%, respectively.

Carry over of diagnostic markers were not observed either as memory effects or ghosting.

As an example, results for a patient with MCD are summarized in Fig. 1 and Table 5. Fig. 1 shows the total ion chromatogram, and Table 5 provides the quantitative results and the comparison to the cut-off value. For all evaluated patient samples the chemical diagnostic results are summarized in Table 6. These results were similar to those of the chemical diagnosis by the conventional method and the clinical symptom.

4. Discussion

In this study, we simplified the solvent extraction and the TMS derivatization of the conventional method [7] by considering the advantages including the appropriate sample clean-up and the established data interpretation [7,16,17]. For sample preparations, aside from liquid extraction and direct/urease [19,20] methods have been developed. Among these, the urease method developed by Matsumoto and Kuhara [20] is the simplest and most efficient in reducing the volume of urine sample and organic solvent. This includes urease treatment to remove urea, deproteinization with ethanol, evaporation to dryness and trimethylsilylation. Another distinctive feature of this method is that both amino acids and sugars can be extracted from urine together with organic acids and

Table 3
Cut-off value obtained from forty normal controls.[a].

No.	Compound[b]	R.I.[c]	m/z		Peak area ratio (%)			
			Q-ion[d]	C-ion[d]	Mean[e]	SD[e]	Range[e]	Cut-off[f]
1	Methylmalonic acid-diTMS	1219	247	218	3.48	2.00	0.00–9.93	9.49
2	Propionylglycine-diTMS*	1359	188	159	0.63	0.87	0.00–3.36	3.23
3	Propionylglycine-diTMS*	1428	260	232	0.00	0.00	0.00–0.00	0.50
4	Glutaric acid-diTMS	1404	261	158	6.39	3.78	1.85–15.85	17.74
5	Isovalerylglycine-TMS*	1488	216	189	1.03	1.67	0.00–6.73	6.03
6	Isovalerylglycine-diTMS*	1520	288	176	0.00	0.00	0.00–0.00	0.50
7	3-Methylcrotonylglycine-TMS*	1564	214	229	0.00	0.00	0.00–0.00	0.50
8	3-Methylcrotonylglycine-diTMS	1578	286	184	0.00	0.00	0.00–0.00	0.50
9	3-Hydroxyglutaric acid-triTMS	1582	247	349	0.00	0.00	0.00–0.00	0.50
10	2-Hydroxyglutaric acid-triTMS	1583	349	203	1.20	0.64	0.00–2.79	3.13
11	Methylcitric acid-tetraTMS*	1862	389	479	0.00	0.00	0.00–0.00	0.50
12	Methylcitric acid-tetraTMS*	1874	389	479	0.00	0.00	0.00–0.00	0.50
13	Margaric acid-TMS	2146	327	145	100.00	0.00	100.00–100.00	100.00
14	Tetracosane	2400	99	67	71.53	13.15	55.75–125.60	110.97

[a] Healthy children less than 3 months.
[b] An asterisk indicates that two TMS derivatives were formed from an organic acid.
[c] R.I. = retention index.
[d] Q- and C-ions were used for selected ion monitoring (SIM).
[e] Mean, SD and range were calculated based on the peak area ratio (TMS derivatives to MGA, %) for 40 normal controls.
[f] Cut-off value was defined as the mean plus three standard deviations of peak area ratio (target compound to MGA).

used for chemical diagnosis. However, large amounts of impurities are extracted simultaneously, thus complicating the organic acid peak identification. On the other hand, the solvent extraction method enables a rapid and accurate identification and prevents column and ion source contamination by removing impurities including sulfur and phosphoric acid originated from urine. This is one of the reasons why the solvent extraction method has been widely used in clinical laboratories [5,6,21]. Therefore, we simplified the solvent extraction procedure used in the conventional method.

To simplify the solvent extraction, reduction of the volume of urine sample and the number of extractions performed is the most effective if the organic acids are to be extracted quantitatively and detected. In the new method, the volume was reduced from 2 ml to 0.2 ml and the volume of extraction solvent (ethyl acetate) was proportionally reduced from 12 ml to 1.2 ml. The number of extractions performed was reduced from two to one.

Moreover, the TMS derivatives of diagnostic markers for the evaluated acidurias could be detected without oximation process in the conventional method. However, this method cannot be applied to the chemical diagnosis in which keto acids are indispensable as the diagnostic markers, for example, succinylacetone for tyrosinemia type I or branched-chain alpha-keto acids for maple syrup urine disease.

To simplify the TMS derivatization, the flash-heater derivatization was applied; this technique is widely used for methylation of drugs [9], TMS derivatization of morphine [10] and TMS derivatization of fatty acid [11,22,23]. In the new method, a 1 μl aliquot of the extract (ethyl acetate) was sandwiched between two plugs of the BSTFA + 1%TMCS in the injection syringe and injected into the glass liner with glass wool at 280 °C as mentioned in Section 2.2.2 (Flash-heater derivatization). The organic acids were derivatized in the glass liner and the front part of the capillary column immediately before gas chromatographic analysis. On the other hand, in the conventional method, the extraction solvent of approximately 12 ml is evaporated under a stream of nitrogen gas and the residue was trimethylsilylated with 0.1 ml of BSTFA + 1%TMCS for 30 min at 80 °C. As the results, the evaporation step after the extraction could be omitted and the volume of BSTFA + 1%TMCS could be greatly reduced for each analysis (approximately 1/70 reduction) by using

Table 4
Quantitative results of TMS derivatives of diagnostic markers in urine sample obtained from patients.

Sample[a]	Compound[b]	Peak area ratio (%)[c]		
		Mean	SD	%RSD
MMA	Methylmalonic acid-diTMS	1456.1	116.4	8.0
PPA	Propionylglycine-TMS*	47.2	2.0	4.3
	Propionylglycine-diTMS*	0.0	0.0	–
	2-Hydroxyglutaric acid-triTMS	16.7	0.6	3.4
	Methylcitric acid-tetraTMS* (1)[d]	6.6	0.5	7.0
	Methylcitric acid-tetraTMS* (2)[d]	4.1	0.4	9.6
GAI	Glutaric acid-diTMS	4568.4	369.3	8.1
	3-Hydroxyglutaric acid-triTMS	21.3	2.5	12.0
IVA	Isovalerylglycine-TMS*	1010.6	51.0	5.1
	Isovalerylglycine-diTMS*	0.0	0.0	–
MCD	3-Methylcrotonylglycine-TMS*	6.3	0.7	11.6
	3-Methylcrotonylglycine-diTMS*	0.0	0.0	–

[a] Sample was obtained from patients diagnosed by the conventional method and clinical symptom. *Abbreviations of diseases*: MMA, methylmalonic acidemia; PPA, propionic acidemia; GAI, glutaric aciduria type I; IVA, isovaleric acidemia; MCD, multiple carboxylase deficiency.
[b] An asterisk indicates that more than two TMS derivatives were formed form an organic acid.
[c] Quantitative results are expressed in the form of the peak area ratio (TMS derivatives to MGA, %). Mean, SD and %RSD were calculated by five analyses.
[d] Methylcitric acid-tetraTMS (1) and (2) are isomers.

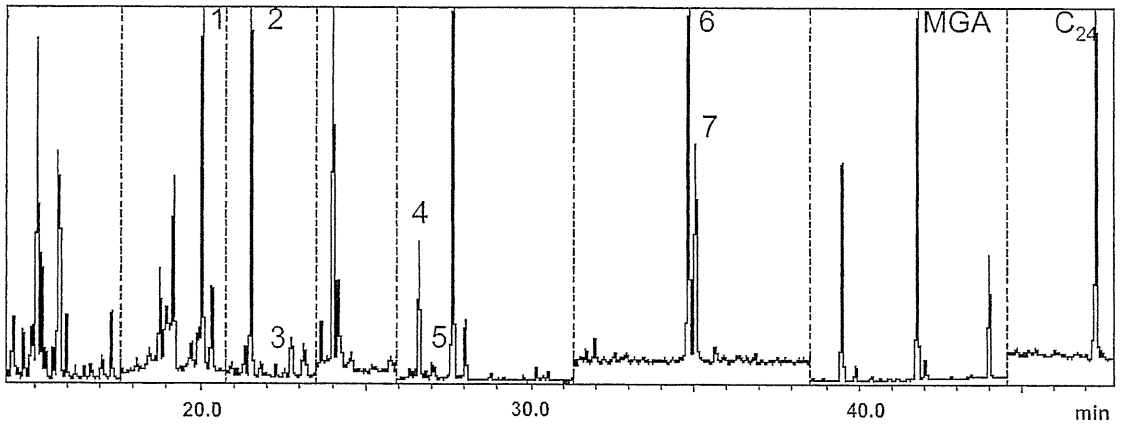

Fig. 1. Total ion chromatogram of SIM of urine sample obtained from a patient with multiple carboxylase deficiency using the new method. Using the new method, urine sample obtained from a patient with multiple carboxylase deficiency was analyzed. The following target compounds were detected: (1) propionylglycine-TMS; (2) glutaric acid-diTMS; (3) propionylglycine-diTMS; (4) 3-methylcrotonylglycine-TMS; (5) 3-methylcrotonylglycine-diTMS; (6) methylcitric acid-tetraTMS; (7) methylcitric acid-tetraTMS; MGA = margaric acid-TMS (I.S.); C_{24} = tetracosane (I.S.). I.S.: internal standard.

the new method. The chance of exposing the operator to extraction solvent and derivatization reagent could also be minimized.

The evaluated organic acids added to urine sample, which formed only one derivative, showed excellent repeatability (%RSD less than 10.8%) (Table 2). These results indicate that those organic acids were extracted quantitatively and completely derivatized by the new method. However, the minimum ratio of the relative recoveries (the new to conventional methods) was 0.27 (4-hydroxyphenyllactic acid-triTMS). In order to cope with the lower recovery, selected ion monitoring mode (SIM) was used for the mass spectrometer measurement.

3-Hydroxyisovaleric acid, 2-hydroxyisovaleric acid, phenyllactic acid and isovalerylglycine formed two TMS derivatives in both methods (Table 2). With the new method, the main products such as 3-hydroxyisovaleric acid-TMS and isovalerylglycine-TMS tended to form derivatives with fewer TMS-groups in contrast to the conventional method, which produced derivatives with more TMS-groups as the main product such as 3-hydroxyisovaleric acid-diTMS and isovalerylglycine-diTMS. In spite of the number of TMS-groups, the repeatabilities of the main products were less than 7.4% in the new method and the ratios of relative recoveries were much higher than those of the by-products. Additionally, the carryover of diagnostic markers was not detected for the patients' sample which contained diagnostic markers at high concentrations. These results indicate that those organic acids were also extracted and derivatized quantitatively.

In the patients' urine analysis (Table 4), the repeatabilities of the main TMS derivatives of the diagnostic markers were excellent (%RSD not over 12%) similar to the analysis of urine sample spiked with representative organic acids (Table 2).

From these results, it can be concluded that the new method could be applied to the chemical diagnosis.

As described above, when multiple TMS derivatives were formed, the main TMS derivative could be chosen as the TMS derivatives of the diagnostic marker (the target compound) similar to the conventional method. Propionylglycine-diTMS (related to PPA), isovalerylglycine-diTMS (to IVA) 3-methylcrotonylglycine-diTMS (to MCD), which are the TMS derivatives of the diagnostic markers

Table 5
Quantitative results[a] of urine obtained from a patient with multiple carboxylase deficiency.

No.	Compound[b]	Patient	Control[c] (%) (n = 40)			
			Mean	Range	Cut-off	Factor[d]
1	Methylmalonic acid-diTMS	8.10	3.48	0.00–9.93	9.49	0.85
2	Propionylglycine-TMS*	27.32	0.63	0.00–3.36	3.23	8.45*
3	Propionylglycine-diTMS*	0.69	0.00	0.00–0.00	0.50	1.38*
4	Glutaric acid-diTMS	42.95	6.39	1.85–15.85	17.74	2.42*
5	Isovalerylglycine-TMS*	0.00	1.03	0.00–6.73	6.03	0.00
6	Isovalerylglycine-diTMS*	0.00	0.14	0.00–5.75	2.87	0.00
7	3-Methylcrotonylglycine-TMS*	30.30	0.00	0.00–0.00	0.50	60.60*
8	3-Methylcrotonylglycine-diTMS*	5.63	0.00	0.00–0.00	0.50	11.27*
9	3-Hydroxyglutaric acid-triTMS	0.00	0.00	0.00–0.00	0.50	0.00
10	2-Hydroxyglutaric acid-triTMS	1.33	1.20	0.00–2.79	3.13	0.42
11	Methylcitric acid-tetraTMS* (1)[e]	2.92	0.00	0.00–0.00	0.50	5.84*
12	Methylcitric acid-tetraTMS* (2)[e]	1.91	0.00	0.00–0.00	0.50	3.83*
13	Margaric acid-TMS	100.00	100.00	100.00–100.00	100.00	1.00
14	Tetracosane	74.99	71.53	55.75–125.60	110.97	0.68

Interpretation:
Multiple carboxylase deficiency

[a] Quantitative results are expressed in the form of the peak area ratio (TMS derivatives to MGA, %).
[b] An asterisk indicates that two TMS derivatives were formed form an organic acid.
[c] Mean, range and cut-off values based on the peak area ratio (TMS derivatives to MGA) were obtained from 40 normal controls (Table 3).
[d] Factor is the ratio of the measured to the cut-off values. If the measured value was more than the cut-off value, the compounds judged as abnormal and marked with an asterisk.
[e] Methylcitric acid-tetraTMS (1) and (2) are isomers.

Table 6
Chemical diagnostic results by the new method.

Patient[a]	Diagnostic result		Compound name[b]	Mark[c]	Factor range[d]
	Conventional[e]	New[f]			
MMA	4	4	Methylmalonic acid-diTMS	4	3.52–326.58
PPA	4	4	Propionylglycine-TMS	1	0.00–15.28
			Methylcitric acid-tetraTMS (1)[g]	4	2.71–25.61
			Methylcitric acid-tetraTMS (2)[g]	4	3.15–15.33
GAI	5	5	Glutaric acid-diTMS	5	1.42–654.15
			3-Hydroxyglutaric acid-triTMS	5	17.60–58.60
IVA	3	3	Isovalerylglycine-TMS	3	4.45–297.32
MCD	4	4	3-Methylcrotonylglycine-TMS	4	60.60–158.56
			3-Methylcrotonylglycine-diTMS	4	11.27–42.01
			Methylcitric acid-tetraTMS (1)[g]	3	1.08–5.84
			Methylcitric acid-tetraTMS (2)[g]	4	1.13–4.29

[a] *Abbreviations of diseases*: MMA, methylmalonic acidemia; PPA, propionic acidemia; GAI, glutaric aciduria type I; IVA, isovaleric acidemia; MCD, multiple carboxylase deficiency.
[b] TMS derivatives used as the target compounds for the chemical diagnosis.
[c] The number of samples which gave a higher quantitative result than the cut-off value for each target compound.
[d] Factor range is the range of ratio (the measured to the cut-off values) for the target compound. If the ratio was more than one, the compounds judged as abnormal.
[e] The number of patients diagnosed by the conventional method and clinical symptom.
[f] The number of patients diagnosed by the new method. Both the conventional and new methods showed the same diagnostic results for all patients.
[g] Methylcitric acid-tetraTMS (1) and (2) are isomers.

by the conventional method, were not detected by the new method (Table 4). However, propionylglycine-TMS, isovalerylglycine-TMS and 3-methylcrotonylglycine-TMS were detected as the corresponding TMS derivatives and the repeatabilities (%RSDs) obtained by the new method were less than 12.0%. The main products of acylglycines in the new method were TMS derivatives with fewer TMS-groups because the reaction time in the liner was shorter than that in the conventional method. Based on these results, it can be concluded that these TMS derivatives could be used for chemical diagnosis.

As the TMS derivatives and their relative recoveries obtained by using the new method were not the same as those obtained by using the conventional method (Table 2), the cut-off value for the conventional method could not be applied to the new method. Therefore, the cut-off values should be determined from the analysis of normal controls (Table 3).

Both the new method and the estimated cut-off values (Table 3) were successfully applied to the chemical diagnoses of 20 patients. For example, MCD is a disorder of biotin metabolism, resulting in impaired activities of the four biotin-dependent carboxylases: propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase and acetyl-CoA carboxylase. The urinary organic acid analysis of the patient revealed an elevation of 3-methylcrotonylglycine and methylcitric acid, and an absence of methylmalonic acid [7]. The results from the quantitative analysis of urine obtained from a patient with MCD showed that 3-methylcrotonylglycine and methylcitric acid were detected as higher than the cut-off values and methylmalonic acid was detected as lower than the cut-off values (Table 5). Although 3-methylcrotonylglycine-diTMS was not detected in another patient's urine in Table 4, it was detected in this patient's urine owing to the higher concentration. Therefore, by using the new method, we could diagnose that the patient had MCD, which was also the chemical diagnosis obtained from the conventional method and the clinical symptoms. For the other patients with the five different diseases, the chemical diagnosis results agreed with those of the conventional method and their clinical symptoms (Table 6). Although the %RSDs in the new method are larger than those in the conventional method in evaluated samples, these results showed that the new method can be applied to the chemical diagnosis of methylmalonic acidemia, propionic acidemia, isovaleric acidemia, glutaric aciduria type I and multiple carboxylase deficiency. Further studies may be needed before the new method can be applied to routine analysis, especially for the cases where lower analytical errors are required [24], such as for therapy monitoring and for the diagnosis of patients as having moderate hyper-excretions or not in an acute episode.

Since the organic acids were not concentrated in the new method, SIM was used for mass spectrometry to improve sensitivity [24]. All urine samples from patients could be analyzed without missing the target compounds. However, the concentrations of target compounds excreted to urine depended on the patient's condition and the enzyme activity. In order to eliminate any undetectable target compounds at trace levels of concentration and prevent the carryover of higher concentration levels, the concentration range must be confirmed by analyzing more real samples.

In future, we will expand this method to other organic aciduria by confirming recovery and repeatability and estimating the cut-off value.

5. Conclusion

This new method enables simple, rapid and safe sample preparation for urinary organic acid analysis using GC/MS by reducing the total volume of organic solvent and derivatization reagents and by the flash-heater derivatization technique. It was successfully applied to 20 patients with the 5 organic acidurias, providing the same chemical diagnosis results as the conventional method. This method will be useful for the chemical diagnosis of organic acidurias and also the clinical applications of urinary organic analysis because of easy and safe sample preparations.

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A common mutation, R208X, identified in Vietnamese patients with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency

Toshiyuki Fukao [a,b,*], Hoan Thi Nguyen [c], Nhan Thu Nguyen [c], Dung Chi Vu [c], Ngoc Thi Bich Can [c], Anh Thi Van Pham [c], Khanh Ngoc Nguyen [c], Hironori Kobayashi [d], Yuki Hasegawa [d], Thao Phuong Bui [c], Kary E. Niezen-Koning [e], Ronald J.A. Wanders [f], Tom de Koning [g], Liem Thanh Nguyen [c], Seiji Yamaguchi [d], Naomi Kondo [a]

[a] Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu 501-1194, Japan
[b] Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1194, Japan
[c] National Hospital of Pediatrics, Hanoi, Viet Nam
[d] Department of Pediatrics, Faculty of Medicine, Shimane University, Izumo, Shimane 693-8501, Japan
[e] Department Laboratory Medicine, Section Metabolic Diseases, University Medical Center Groningen, Groningen, University of Groningen, 9713 GZ Groningen, The Netherlands
[f] Laboratory Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands
[g] Department of Pediatric Metabolic Diseases, University Medical Centre Utrecht, 3508 AB Utrecht, The Netherlands

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ABSTRACT

Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency is an inborn error of metabolism affecting isoleucine catabolism and ketone body utilization. This disorder is clinically characterized by intermittent ketoacidotic episodes with no clinical symptoms between episodes. In general, T2 gene mutations are heterogenous. No common mutations have been identified and more than 70 mutations have been identified in 70 patients with T2 deficiency (including unpublished data). We herein identified a common mutation, R208X, in Vietnamese patients. We identified R208X homozygously in six patients and heterozygously in two patients among eight Vietnamese patients. This R208X mutation was also identified heterozygously in two Dutch patients, however, R208X mutant alleles in the Vietnamese have a different haplotype from that in the Dutch, when analyzed using Msp I and Taq I polymorphisms in the T2 gene. The R208X mutant allele was not so frequent in the Vietnamese since we could not find that mutant allele in 400 healthy Vietnamese controls using the Nla III restriction enzyme assay. DNA diagnosis of T2 deficiency may be applicable to the Vietnamese population.

Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9, gene symbol *ACAT1*) deficiency (OMIM 203750, 607809) is an autosomal recessive disorder, commonly known as β-ketothiolase deficiency. Since 1971 [1], more than 90 patients with it have been identified (including personal communications) [2]. This disorder is clinically characterized by intermittent ketoacidotic episodes with no clinical symptoms between episodes. T2 plays a role in ketolysis in extrahepatic tissues. T2 also catalyzes thiolysis of 2-methylacetoacetyl-CoA in isoleucine catabolism. Hence, T2-deficient patients usually have urinary excretion of 2-methyl-3-hydroxybutyrate, 2-methylacetoacetate and tiglylglycine, which are hallmarks derived from intermediates in isoleucine catabolism. The severity of the clinical features varies from patient to patient but follow-up studies reveal that, in general, T2 deficiency has a favorable outcome [3].

Human T2 cDNA is about 1.5 kb long and encodes a precursor protein of 427 amino acids, including a 33-amino-acid leader polypeptide [4]. The T2 (*ACAT1*) gene spans approximately 27 kb, and contains 12 exons [5]. We have identified more than 70 gene mutations ([6–23] and unpublished data). In general, T2 gene mutations are heterogenous and many patients have unique mutations. Several mutations have been identified in more than two independent families, but as far as we know, no common mutations have yet been identified in T2 deficiency.

We herein report identification of a common mutation, R208X, in Vietnamese patients.

Abbreviation: T2, mitochondrial acetoacetyl-CoA thiolase.

* Corresponding author. Address: Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan. Fax: +81 58 230 6387.
E-mail addresses: toshi-gif@umim.net, toshi-gif@umin.ac.jp (T. Fukao).

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Materials and methods

Vietnamese patients

Ten Vietnamese patients (who were not related to each other) from the northern half of Vietnam (around Hanoi) were suspected of having T2 deficiency from urinary organic acid analysis in Shimane University from 2005 to 2009. All patients belonged to the major Vietnamese ethnic group, the Kinh. 2-Methyl-3-hydroxybutyrate and tiglylglycine were detected in their urinary organic acid profiles. A typical acylcarnitine profile of elevated levels of C5:1 and C5OH was also detected in the nine patients analyzed. Among them, samples from eight patients were available for this study. All eight had developed severe ketoacidotic crises and were referred to the National Hospital of Pediatrics in Hanoi for intensive care or evaluation. Their clinical presentations are summarized in Table 1. Among the eight patients, GK74 died at 25 months due to a severe second ketoacidotic crisis after confirmation of chemical diagnosis at 18 months. GK70 experienced only one ketoacidotic crisis at 30 months which was severely complicated by delayed mental development with convulsions, hypotonia and DQ 60. The other cases have achieved normal development thus far.

Dutch patients

GK36 is a Dutch girl born from non-consanguineous parents in 1994. The parents are Dutch Caucasians. She was consulted for evaluation of motor skills and suspected of having T2 deficiency at the age of 1 y 10 m by urinary organic acid analysis. 2-Methyl-3-hydroxybutyrate, 2-methylacetoacetate, and tiglylglycine were detected in her urine in asymptomatic condition. She did not experience severe ketoacidotic crisis until the age of 13 y 7 m. Her development is now normal.

GK35 was evaluated at the age of 19 months because of recurrent episodes of hypoglycemia with metabolic acidosis. He is the third child of healthy non-consanguineous Dutch parents. Urinary organic acid analysis prompted the diagnosis of T2 deficiency. He was treated with a mild protein restriction in infancy, as well as L-carnitine. Psychomotor development has been uneventful and episodes of hypoglycemia and acidosis have not reccured. At present the patient is nearly 14 years old.

Urinary organic acid analysis

Urinary organic acid analyses for these Vietnamese patients were done in Shimane university using dried filter paper, as described in Ref. [24].

Mutation detection

This study was approved by The Ethical Committee of The Graduate School of Medicine, Gifu University. Genomic DNA in the Vietnamese patients and their families was purified from blood with QIAamp DNA blood mini kits (Qiagen Inc., Valencia, CA, USA). Genomic DNA from the Dutch patients was extracted from fibroblasts using SepaGene kit (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed at the genomic level by PCR and direct sequencing using a primer set for fragments, including an exon and its intron boundaries [11].

Restriction enzyme assay to detect R208X

The R208X substitution (CACG to CATG) creates a new Nla II site.

Table 1
Clinical and molecular summary of T2-deficient patients.

	Consanguinity	Sex	Death of elder siblings	The first crisis								Prognosis			Mutations		Polymorphisms	
				Onset of 1st crisis	Preceding illness	pH	HCO3	BE	Glucose	NH3	Unconsciousness	Poly-pnea	Mechanical ventilation	Number of crises	Present age	Condition	Paternal allele	Maternal allele	Msp I P	Taq I P
Vietnamese																				
GK70	–	M		34 m	Pneumonia				5.5	196	Coma	+	+	1	4.5	Delayed	R208X	163_167del 5ins2	Mm	Tt
GK72	–	M	Sis 24 m	12 m	Pneumonia	6.93	4	−26	2		Coma	+		2	4	Good	R208X	R208X	MM	TT
GK73	–	M	Bro 11 m	13 m		7.08	2.4	−27	5.8	86	Coma	+		1	4	Good	R208X	R208X	MM	TT
GK74	–	M		18 m	Pneumonia	6.9	2	−20	5.9	96	Coma	+	+	2	Died at 2 y		IVS10-1g>c	R208X	Mm	Tt
GK75	–	M	Bro 27 m	11 m	Acute diarrhea	6.88	1.5		5.3	39.4	Lethargy	+		1	2	Good	R208X	R208X	MM	TT
GK76	–	M		13 m		7.11	3.3	−26	19.4		Coma	+		2	4	Good	R208X	R208X	MM	TT
GK79	–	F		12 m	Acute diarrhea	7.1	4	−20	6		Lethargy	+		2	3.5	Good	R208X	R208X	MM	TT
GK80	–	F		9 m	Acute diarrhea	6.89	2.6	−28	5.5	130	Coma	+	+	1	1	Good	R208X	R208X	MM	TT
Dutch																				
GK35	–	M		12 m	Pneumonia	7.13	8	−20.5	3		Lethargy	+		2	14	Good	R208X	IVS11+2t>c	mm	Tt
GK36	–	F		No acute episodes										0	13	Good	R208X	IVS10-2a>c	mm	Tt

GK79 was evaluated in stable condition after two acute acidotic episodes and diagnosed as having T2 deficiency.
GK36 was suspected of having T2 deficiency by organic acid analysis performed for evaluation of motor skills at the age of 1 y 9 m.
Deaths of elder siblings due to metabolic decompensation were noted in 3 patients: Sis 24 m means a sister died at 24 months of age.
* In Dutch patients, familial segregation of these mutations was not determined.

A fragment (264 bp), including exon 7 and its surrounding introns, was amplified using the following primers:

In6s (in intron 6, −79 ~ −60) 5′-CACTATAAGTTAGGCAAAGT-3′

In7as (in intron 7, +39 ~ +20) 5′-TGAAAAGTCTATTCATCCTT-3′

After PCR amplification, an aliquot of an amplicon was digested with Nla III, then subjected to a 5% polyacrylamide gel.

R208X mutant allele in Vietnamese population

We used the above restriction enzyme assay to detect the R208X mutant allele in 400 healthy Vietnamese controls. The fragment which included exon 7 was amplified from a blood filter 1.25 mm in diameter using Amplidirect Plus (Shimadzu Biotech, Tsukuba, Japan).

Msp I and Taq I polymorphisms

There are two well-known polymorphisms in the T2 gene. One is c.13G/C in exon 1, which can be detected by the absence/presence of the Msp I site [7]. The other is IVS9+84C>T, which can be detected by the presence/absence of the Taq I site [25]. These polymorphic sequences were determined by direct sequencing.

Results and discussion

Identification of gene mutations in Vietnamese patients

From 2005 to 2009, 10 Vietnamese patients (who were not related to each other) from the northern half of Vietnam (around Hanoi) were suspected of having T2 deficiency from typical profiles of urinary organic acids (Fig. 1). In this report, eight of the 10 patients were investigated at the DNA level. Their clinical presentations are summarized in Table 1. The National Hospital of Pediatrics in Hanoi covers an area of about 40 million people in the northern part and some middle parts of Vietnam. Since most of the very sick children are referred to this hospital, most T2-deficient patients who develop severe ketoacidotic crisis are expected to be examined in this hospital. This area has approximately 555,000 newborns per year. The birth years of the 10 T2-deficient patients were from 2003 to 2008. Hence, if all the patients with T2 deficiency in this area were identified in this hospital, the incidence of T2 deficiency is calculated to be about 1 in 333,000 newborns in this area (10 T2-deficient patients/555 × 6 years). Some patients may die before referral to this hospital, so the incidence of T2 deficiency may be more than this value.

We first confirmed T2 deficiency in GK70's fibroblasts. Acetoacetyl-CoA thiolase activities with and without potassium ions were 3.5 and 3.7 nmol/min/mg protein (4.8 and 10.1 in control fibroblasts), respectively, showing no potassium ion-activated acetoacetyl-CoA thiolase activity. This indicated that GK70 had T2 deficiency. Immunoblot analysis showed that the T2 protein was not detectable in GK70's fibroblasts (data not shown). Mutation analysis at the genomic level showed that GK70 was a compound heterozygote of c.622C>T (R208X) from the father and c.163_167delTTTTTinsAA from the mother. The latter mutation resulted in F55del and L56K. This mutation was not identified in 50 control Vietnamese subjects.

The other 7 Vietnamese patients who were suspected of having T2 deficiency from urinary organic acid analysis were analyzed using DNA samples. In the cases of GK72, GK73, GK74, and GK75, we sequenced all exons and their surrounding introns (~100 bp) and identified the mutations shown in Table 1. Fig. 2A shows the result of direct sequencing of the fragment, including exon 7, in GK73 and his parents. GK73 was a homozygote of R208X. Since the R208X mutation was identified in eight of 10 mutant alleles in these 5 Vietnamese patients (GK70, GK72–75), in cases of GK76, GK79, and GK80, we screened the presence of R208X first and revealed that the three patients were homozygotes of R208X.

Allele frequency of R208X in Vietnamese population

Since these 8 families with T2 deficiency were not related to each other, we expected that the R208X mutant allele would be prevalent in the Vietnamese population. We screened the R208X mutation in 400 Vietnamese healthy subjects, using the restriction enzyme assay (Fig. 2b). We could not identify R208X in 800 Vietnamese alleles. This may indicate that the frequency of R208X homozygous T2-deficient patients in a Vietnamese population is less than 1/640,000. As discussed above, we first expected to detect some heterozygotes of the R208X mutation when we examined 800 alleles, but this time we could detect no heterozygote of R208X.

Identification of gene mutations in Dutch patients

We previously identified the c.622C>T(R208X) mutations heterozygously in two Dutch patients (GK35 and GK36). As shown in Table 1, GK35 and GK36 were compound heterozygotes of R208X and IVS11+2t>c, and R208X and IVS10-2a>c. We previously identified mutations in the other 3 Dutch patients as follows: GK04 was a compound heterozygote of G183R and IVS10-2a>c; GK04's father, GK05, was a compound heterozygote of G183R and IVS8+1g>t [6]; GK17 was a compound heterozygote of IVS7-46_c.752del68bp and IVS11+2t>c [9]. Hence among 9 mutant

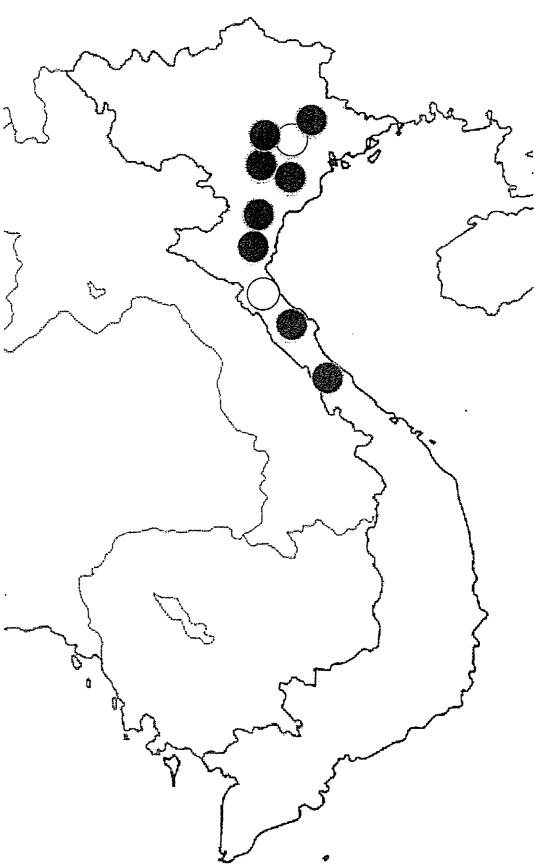

Fig. 1. T2-deficient patients identified in Vietnam. A closed circle indicates a T2-deficient patient whose mutations were confirmed. One open circle indicates a probable T2-deficient patient whose sample was not available for mutation analysis.

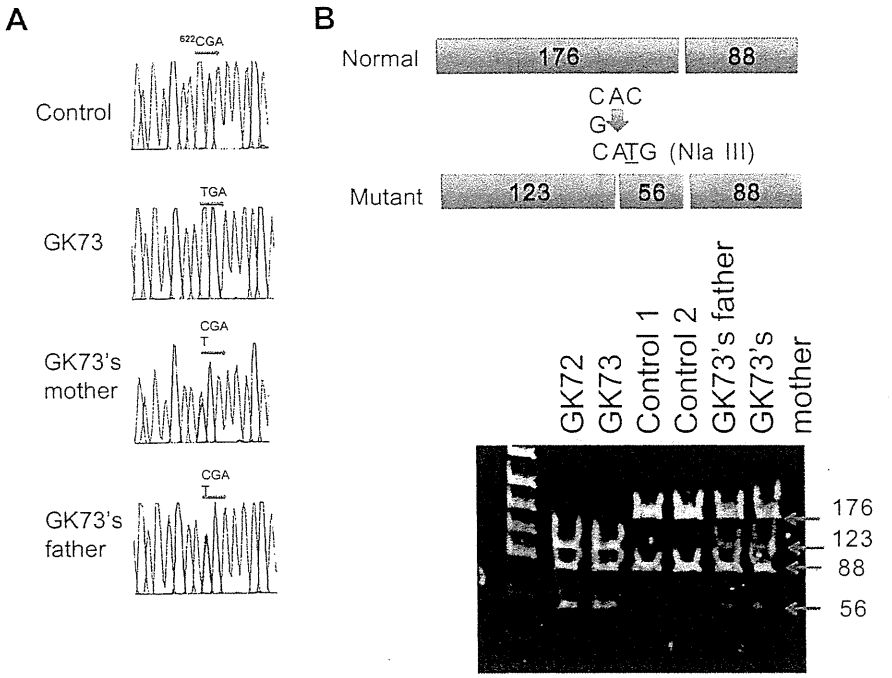

Fig. 2. Identification of R208X mutation. (A) Genomic direct sequencing of exon 7. GK73 had a homozygous c.622C>T (R208X) mutation and the parents were carriers of this mutation. (B) Restriction enzyme assay to detect R208X by Nla III. PCR fragments digested with Nla III were separated on a 5% polyacrylamide gel. Samples from two homozygotes (GK72 and GK73), two controls, and two heterozygotes (GK73's father and mother) are shown as representative data.

alleles in a Dutch population, R208X, IVS11+2t>c, and IVS10-2a>c were identified in two mutant alleles. IVS11+2t>c was also identified in other Caucasian patients (GK09, GK28). This IVS11+2t>c caused aberrant splicing using a cryptic splice site just 4 bp downstream of the authentic site, resulting in a 4-bp insertion to T2 mRNA [7].

Haplotyping of R208X mutant allele in Vietnamese and Dutch populations

Two T2 gene polymorphisms were reported [7,25]. One is c.13G/C in exon 1, which can be detected by the absence/presence of the Msp I site [7]. The heterozygosity of the Msp I polymorphism was reported to be 0.34 on NCBI SNP (http://www.ncbi.nlm.nih.gov/SNP/). The other is IVS9+84C>T, which can be detected by the absence/presence of Taq I site [25]. The heterozygosity of the Taq I polymorphism in Japanese population was reported to be 0.5. As shown in Table 1, the R208X allele in Vietnamese patients had an MT haplotype, which was confirmed by familial analysis. However, since the R208X mutant allele in Dutch patients had an m instead of an M, the haplotype of the R208X mutant allele in Dutch patients was different from that in Vietnamese patients.

These data suggested that the R208X mutation is a founder mutation in the Vietnamese population but independently occurred from R208X in the Dutch population.

Common mutations in T2 deficiency

To date, more than 70 different mutations have been identified in more than 70 T2-deficient patients (including unpublished data). Among the mutations, only a few were identified in more than two independent families: for example c.149delC in the Japanese population [12], c.455G>T(G152A) [18], c.890C>T(Q272X) [8], IVS8+1g>t [6], c. 890C>T(T297M)[10] and IVS11+2t>c [6] in the Caucasian population. As far as we know, no common mutations have been identified in T2 deficiency. However, as described above, the R208X mutation has been identified in 87.5% of mutant alleles in the Vietnamese population. The remarkably high incidence of the R208X mutation among Vietnamese T2-deficient patients is similar to the high incidence of c.727G>T in the G6PC gene among Japanese Glycogen storage disease-type Ia patients, and of the K329E mutation in the ACADM gene among Caucasian medium-chain acyl-CoA dehydrogenase-deficient patients [26–28]. DNA diagnosis of T2 deficiency may be applicable to the Vietnamese population. We have not analyzed mutations in Asian populations other than Vietnam and Japan, hence we do not know whether the R208X mutation is prevalent in Southeast Asia or not. We are planning to examine mutations in other Southeast Asian countries.

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Original article

Effect of heat stress and bezafibrate on mitochondrial β-oxidation: Comparison between cultured cells from normal and mitochondrial fatty acid oxidation disorder children using in vitro probe acylcarnitine profiling assay

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

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

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Abstract

Hyperpyrexia occasionally triggers acute life-threatening encephalopathy-like illnesses, including influenza-associated encephalopathy (IAE) in childhood, and can be responsible for impaired fatty acid β-oxidation (FAO). In this regard, patients with impaired FAO may be more susceptible to febrile episodes. The effects of heat stress and a hypolipidemic drug, bezafibrate, on mitochondrial FAO were investigated using cultured cells from children with FAO disorders and from normal controls, using an in vitro probe acylcarnitine (AC) profiling assay. Fibroblasts were incubated in medium loaded with unlabelled palmitic acid for 96 h at 37 and 41 °C, with or without bezafibrate. AC profiles in culture medium were analyzed by electrospray ionization tandem mass spectrometry. Heat stress, introduced by 41 °C, significantly increased acetylcarnitine (C2) but slightly decreased the other acylcarnitines (ACs) in controls and medium-chain acyl-CoA dehydrogenase (MCAD)-deficient cells. On the other hand, in very long-chain acyl-CoA dehydrogenase (VLCAD)-deficient cells, accumulation of long-chain ACs were enhanced at 41 °C, compared with that at 37 °C. In contrast, bezafibrate decreased long-chain ACs with significant increase of C2 in both control and VLCAD-deficient cells at 37 °C. These data suggest that heat stress specifically inhibits long-chain FAO, whereas bezafibrate recovers the impaired FAO. Our approach is a simple and promising strategy to evaluate the effects of heat stress or therapeutic drugs on mitochondrial FAO.

Keywords: Heat stress; Bezafibrate; Mitochondrial fatty acid β-oxidation disorder; In vitro; Acylcarnitine profiling

1. Introduction

Hyperpyrexia occasionally triggers acute life-threatening encephalopathy-like illnesses, including influenza-associated encephalopathy (IAE) in childhood [1,2], and it has been reported that heat stress can be responsible for impaired fatty acid β-oxidation (FAO) in IAE [3–5]. In this regard, patients with impaired FAO may be more susceptible to febrile episodes. The mitochondrial FAO is a central energy generating process particularly during long fasting, infection or acute metabolic stress, such as hyperpyrexia [6,7]. Patients with inherited mitochondrial FAO disorders occasionally present acute life-threatening symptoms, such as

* Corresponding author. Tel.: +81 853 20 2216; fax: +81 853 20 2215.
E-mail address: seijiyam@med.shimane-u.ac.jp (S. Yamaguchi).

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encephalopathy or cardio-myopathy, due to energy crisis of metabolic decompensation [8,9], which are often remediable [10,11]. In addition, heat stress was considered as a model of thermal injury to the central nervous system (CNS) in a number of research reports [3,4,12].

In recent decades, in vitro probe acylcarnitine (AC) profiling assay was developed to evaluate FAO disorders [13–16]. AC profiles in culture medium after incubating with various fatty acids as substrates were determined by electrospray ionization tandem mass spectrometry (MS/MS). In order to investigate the consequence of heat stress on impaired FAO, we compared the quantitative AC profiles at 37 and 41 °C in cultured fibroblasts from Japanese children with mitochondrial FAO disorders [15,16] as well as from normal controls. Furthermore, we utilized the same approach to assess the effects of bezafibrate, a common hypolipidemic drug, which acts as activator of peroxisome proliferators activated nuclear receptors (PPARs) and up-regulates the expression of genes encoding mitochondrial enzymes [17], on mitochondrial FAO in the same groups. We report here the effects of heat stress and bezafibrate on mitochondrial FAO with the findings of the in vitro probe AC profiling assay.

2. Materials and methods

2.1. Skin fibroblasts

We cultured fibroblasts from 6 controls (healthy volunteers, passages 3–16) and 9 Japanese VLCAD-deficient children (passages 3–14). VLCAD deficiency is clinically divided into three subgroups [15]: (1) a severe form, with early onset (infancy), high mortality, and high incidence of cardio-myopathy; (2) an intermediate form, with onset from infancy to early childhood, low mortality, and high incidence of hypo-glycemia; and (3) a myopathic form (milder form), with frequent onset in later childhood, adolescence or after, and isolated skeletal muscle involvement triggered by exercise, long fasting or other metabolic stresses. In this study, cell lines from VLCAD deficiency, 2 cases of the severe; 3 of the intermediate; and 4 of the myopathic forms, were examined. We also used 3 cell lines from medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (passages 4–9) for reference in the study.

2.2. In vitro probe assay of AC profiling

Fibroblasts were cultured in 75-cm^2 flasks (Iwaki, Tokyo, Japan) containing modified Eagle's minimal essential medium (MEM; Nissui, 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.

Confluent cells were harvested by trypsinization (0.25%-Trypsin/1 mM-EDTA; Nacalai Tesque) and seeded onto 6-well microplates (35 mm i.d.; Iwaki) with fresh above medium (2 mL/per well) until they reached confluence again. Thereafter, 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 experimental substrate A, MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabelled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, or substrate B, medium of substrate A added bezafibrate (0.4 mmol/L; Sigma). The start and the end points of the 96-h incubation are expressed as T_0 and T_{96}, respectively. AC profiles in the culture medium were analyzed at T_{96}.

Cultured cells were incubated with substrate A at 37 or 41 °C for 96 h to determine the effects of heat stress on mitochondrial FAO. Cells were also incubated in substrate B, medium containing bezafibrate and palmitic acid, at 37, and 41 °C to determine the effects of bezafibrate. In a replicate experiment, the supernatants were collected at 24, 48, 72 and 96 h to determine their time course effects on the FAO.

2.3. Quantitative acylcarnitines analysis

ACs in culture medium supernatants were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Briefly, methanol (200 μL) including an isotopically-labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of the supernatant from culture medium, for 30 min. Portions 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 3 N *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) and then the ACs in 10 μL of the aliquots were determined using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

2.4. Protein concentration, cell count and cell viability

Protein concentrations were measured at T_0 and T_{96}, by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) [18]. Cells were enumerated in a model Z1 Coulter Counter (Beckman Coulter Electronics, Luton, UK). The ratio of viable cells at 24, 48, 72 and 96 h of incubation were determined using the modified 3-(4,5-dimethyl-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay [19].

2.5. Data and statistical analysis

The results are expressed as mean ± SD from at least two independent experiments. The AC concentrations

are expressed as nmol/mg protein. Data were statistically analyzed by the one-way analysis of variance (ANOVA) and post hoc LSD 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. Clinical characters of VLCAD-deficient patients

The clinical characteristics of the patients with VLCAD deficiency are summarized in Table 1. Patients 1 and 2 with the severe form both died in infancy. The patients (from No. 3 to 9) with the intermediate or myopathic forms remain alive, although patients classified into the intermediate form had episodes of cardio-myopathy, acute encephalopathy, hypo-glycemia or hyperammonemia.

3.2. AC profiles in VLCAD-deficient cells under heat stress

As shown in Fig. 1, the levels of C2 in all forms of VLCAD deficiency were lower than that of normal controls at 37 °C, and the levels of long-chain ACs, such as C12, C14, C14:1 and C16, were higher in VLCAD deficiency. In particular, the amount of C14 was remarkably elevated in the severe form, while C12 was significantly increased in all forms of VLCAD deficiency.

Control cells exposed to heat stress (41 °C) showed a significant elevation of C2 ($p = 0.012$), but slightly decreased other species from short-chain to medium-chain ACs (Fig. 1B). Similarly, C2 was significantly elevated in the intermediate and myopathic forms of VLCAD-deficient cells incubated at 41 °C compared to 37 °C ($p < 0.01$). Most importantly, accumulation of C16 was significantly enhanced in all the clinical forms of VLCAD deficiency ($p < 0.01$) (Fig. 1B).

Since overall accumulation of long-chain ACs represents the impairment of long-chain FAO rather than individual long-chain ACs, we calculated the ΣLC, the sum of long-chain AC productions (C12 + C14 + C14:1 + C16), and the ratio of ΣLC/C2, which may be more useful to evaluate the capacity of long-chain FAO. Regardless of temperature, the ΣLC and the ratio of ΣLC/C2 values in VLCAD-deficient cells were significantly higher than those of controls ($p < 0.01$), as shown in Fig. 2. The ΣLC was significantly higher at 41 °C than that at 37 °C in VLCAD-deficient cells ($p = 0.043$), among which myopathic form showed the highest ΣLC, suggesting that the long-chain FAO was inhibited by heat stress and the myopathic form is most sensitive to heat stress ($p < 0.01$).

We compared the effects of heat stress between VLCAD-deficient cells (mild form, $n = 4$) and MCAD-deficient cells ($n = 3$) using the same strategy. As shown in Fig. 3, C2 was significantly increased at 41 °C in all VLCAD-, MCAD-deficient cells as well as normal controls. In VLCAD deficiency (Fig. 3A), medium-chain ACs (C6, C8 and C10) decreased, whereas long-chain ACs (C12, C14, C14:1 and C16) elevated at 41 °C. On the other hand, in MCAD deficiency (Fig. 3B), medium-chain ACs (C6 and C8) were also decreased at 41 °C but long-chain ACs did not change. Namely, while medium-chain ACs were down-regulated by heat stress in control, MCAD-, and VLCAD-deficient cells, long-chain ACs were accumulated exclusively in VLCAD deficiency.

3.3. Effect of bezafibrate on FAO disorder

Compared with the basic condition (Fig. 4A), bezafibrate treatment significantly increased the amount of C2 in VLCAD-deficient cells ($p = 0.014$) as well as control cells, while the accumulation of long-chain ACs remarkably decreased ($p < 0.01$) at 37 °C (Fig. 4B). Although the mean values of ΣLC ($p = 0.029$) and the ratio of ΣLC/C2 ($p = 0.015$) remained higher in VLCAD deficiency patients than that in controls (Fig. 5), bezafibrate significantly reduced ΣLC in all the clinical forms to a different extent at 37 °C (Fig. 5A) compared with that without bezafibrate as shown in Fig. 2A. The reduction of

Table 1
Clinical and biochemical characteristics of patients with VLCAD deficiency.

Patient no.	Age at onset	Clinical subgroup	Outcome	Clinical and biochemical findings					
				Cardio-myopathy	Acute encephalopathy	Myalgia or rhabdomyolysis	Hypo-glycemia	Hyper-ammonemia	Hepato-megaly
1	2 m	Severe	Dead (8 m)	+	+	−	+	+	−
2	4 m	Severe	Dead (5 m)	+	+	−	−	+	+
3	1 y4 m	Intermediate	Alive	−	+	−	+	+	+
4	1 y5 m	Intermediate	Alive	−	+	+	−	−	−
5	4 y9 m	Intermediate	Alive	+	−	+	−	−	−
6	1 y	Myopathic	Alive	−	−	+	−	−	−
7	2 y	Myopathic	Alive	−	−	+	−	−	−
8	2 y10 m	Myopathic	Alive	−	−	+	−	−	−
9	8 y	Myopathic	Alive	−	−	+	−	−	−

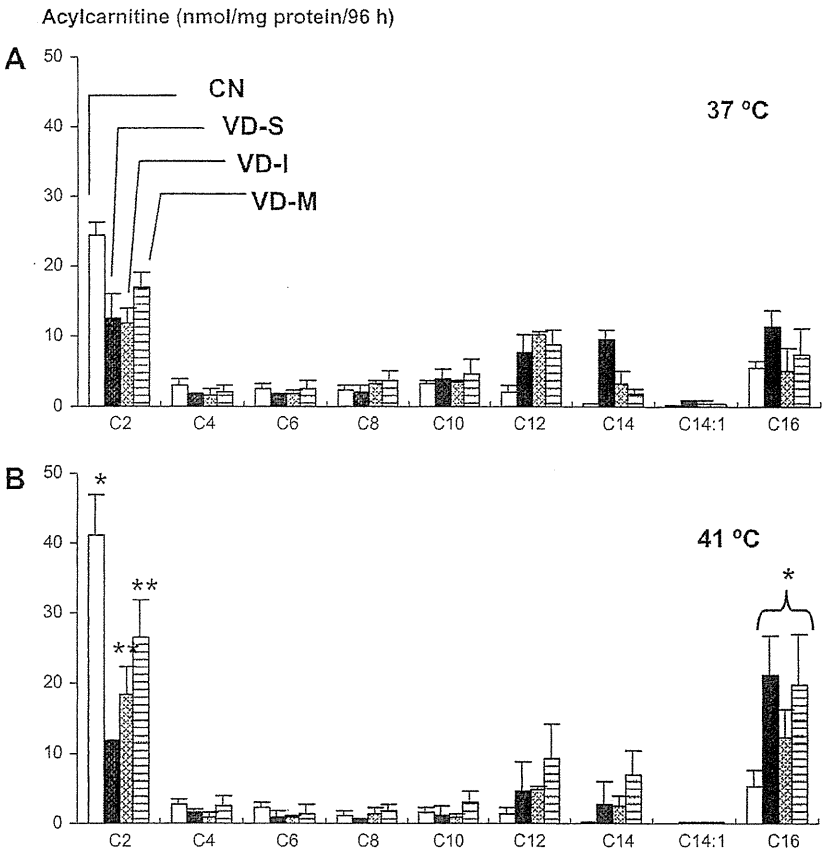

Fig. 1. AC profiles in medium loaded with palmitic acid from VLCAD deficiency with different clinical forms under heat stress. (A) 37 °C; (B) 41 °C. □, Control (CN); ■, severe VLCAD deficiency (VD-S); ▨, intermediate form (VD-I); ▤, myopathic form (VD-M). Data are expressed as mean ± SD (nmol/mg protein/96 h). Significant differences between 37 and 41 °C are shown as $^{**}p < 0.01$ and $^{*}p < 0.05$.

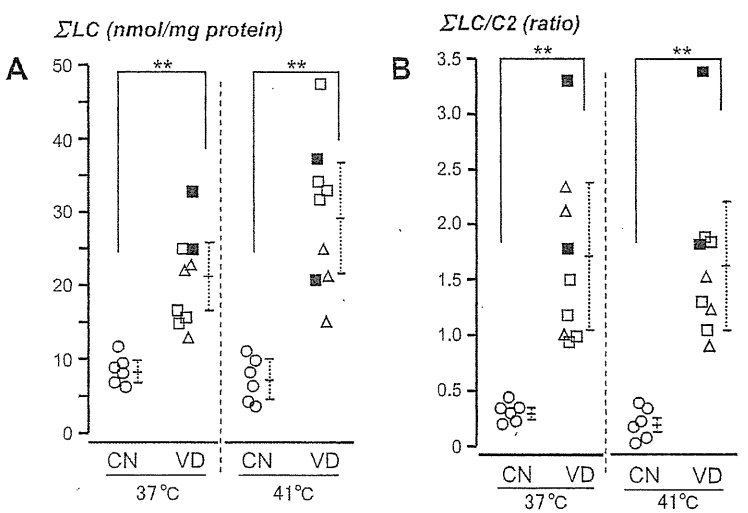

Fig. 2. Comparison of ΣLC and ΣLC/C2 of VLCAD deficiency under heat stress. (A) ΣLC, C12 + C14 + C14:1 + C16; (B) ratio of ΣLC/C2. CN, control; VD, VLCAD deficiency. ○, Control; ■, severe VLCAD deficiency; △, intermediate form; □, myopathic form. Significant differences between different groups are shown as $^{**}p < 0.01$ and $^{*}p < 0.05$.

long-chain ACs tended to be greater in the intermediate (58 ± 24% reduction) and myopathic forms (54 ± 24% reduction) than in the severe form (35 ± 20% reduction). On the other hand, bezafibrate treatment at 41 °C, curiously, reduced all species of AC including C2 in both control and VLCAD-deficient cells (Fig. 4C).

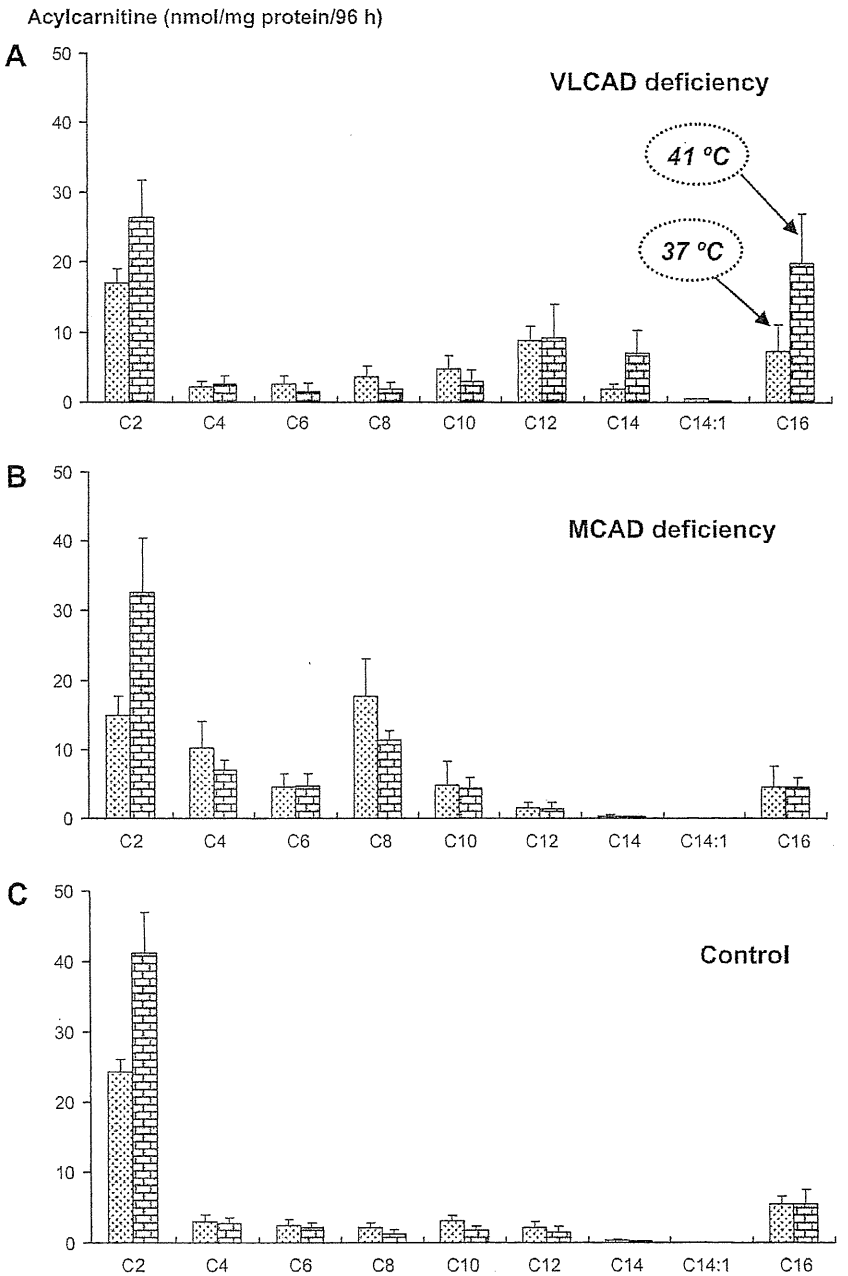

Fig. 3. Comparison of AC profiles in the medium loaded with palmitic acid between VLCAD deficiency and MCAD deficiency under heat stress. (A) VLCAD deficiency; (B) MCAD deficiency; (C) Control. ▨, 37 °C; ▩, 41 °C. Data are expressed as mean ± SD (nmol/mg protein/96 h).

3.4. MTT assay and the protein concentration of cultured cells under different conditions

Since all species of ACs were significantly reduced in the presence of bezafibrate at 41 °C, we measured cell viability using the MTT assay, and protein concentration in lysates to exclude variations in cell number or viability that could otherwise affect ACs in cells cultured in fatty acid-free BSA for up to 96 h under various conditions. The ratio (%) of viable cells (Fig. 6) and the protein concentration (Fig. 7) declined over time in all groups and under all culture conditions. Incubation with bezafibrate at 41 °C diminished the viable cell number after 24 h as shown in Fig. 6 and the average protein concentration at 96-h (T_{96}) was decreased by >50% compared with start point (T_0) as shown in Fig. 7.

4. Discussion

The primary purpose of our study was to investigate the susceptibility of hyperpyrexia, and a hypolipidemic drug, bezafibrate, on mitochondrial FAO capacity using

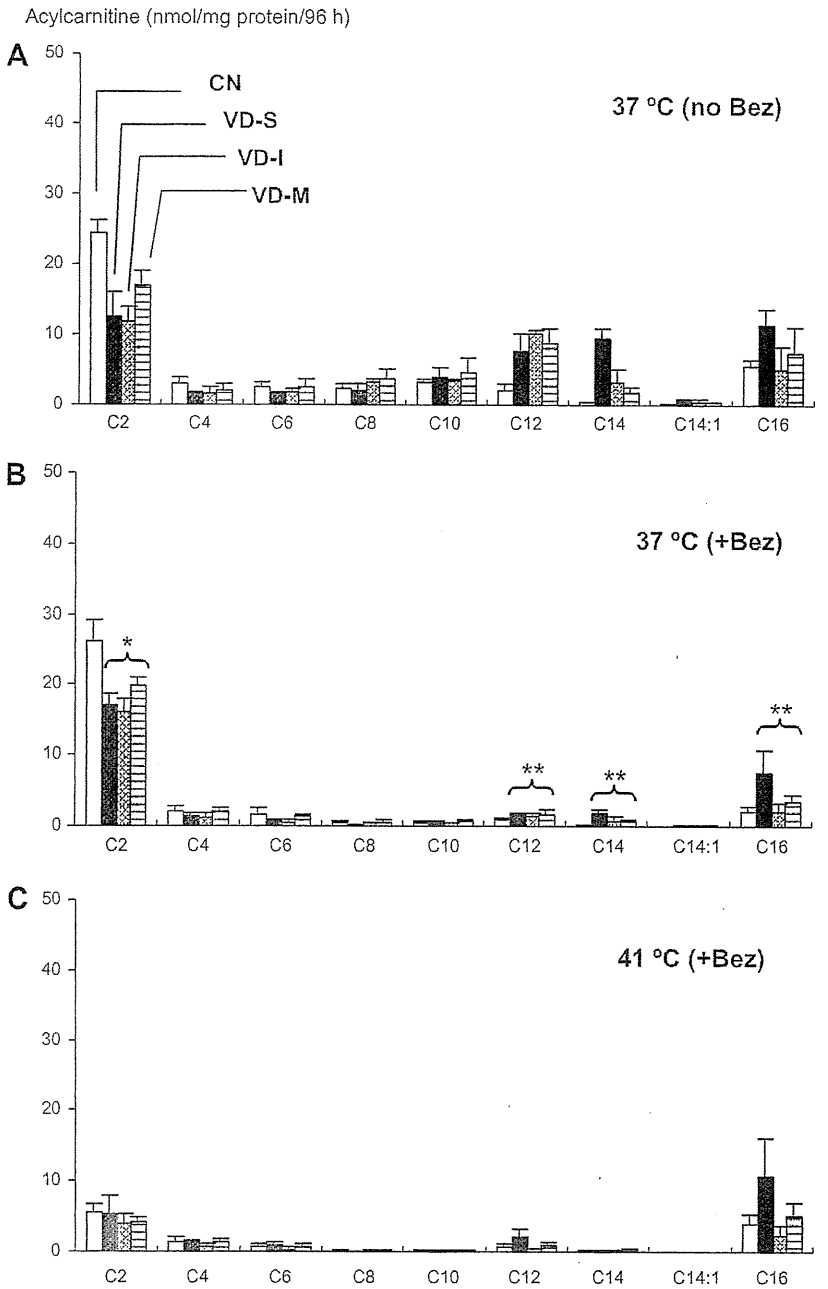

Fig. 4. AC profiles in medium loaded with palmitic acid from VLCAD deficiency with different clinical forms after bezafibrate treatment. (A) 37 °C without Bez; (B) 37 °C + Bez; (C) 41 °C + Bez. □, Control (CN); ■, severe VLCAD deficiency (VD-S); ▨, intermediate form (VD-I); ▤, myopathic form (VD-M). Data are expressed as mean ± SD (nmol/mg protein/96 h). Significant differences between cells treated with or without bezafibrate at 37 °C are shown as $^{**}p < 0.01$ and $^{*}p < 0.05$.

in vitro probe AC profiling assay. Although hyperpyrexia may be responsible for deterioration of various metabolic disorders in childhood and occasionally is associated with life-threatening encephalopathy such as IAE [1–5], it is difficult to confirm the susceptibility to hyperpyrexia in vivo.

We used fibroblasts from patients with VLCAD deficiency, in which longer chain FAO is impaired, as well as normal controls, to determine the effect of heat stress and bezafibrate on mitochondrial FAO. While mitochondrial acyl-CoA dehydrogenases (ACDHs) share the same basic chemical mechanism, they differ markedly in their specificity towards the 'length' of their acyl-CoA substrates [20]. In this context, we evaluated the FAO capacity based on the accumulation of specific length-chain of ACs. Acetylcarnitine (C2), derived from

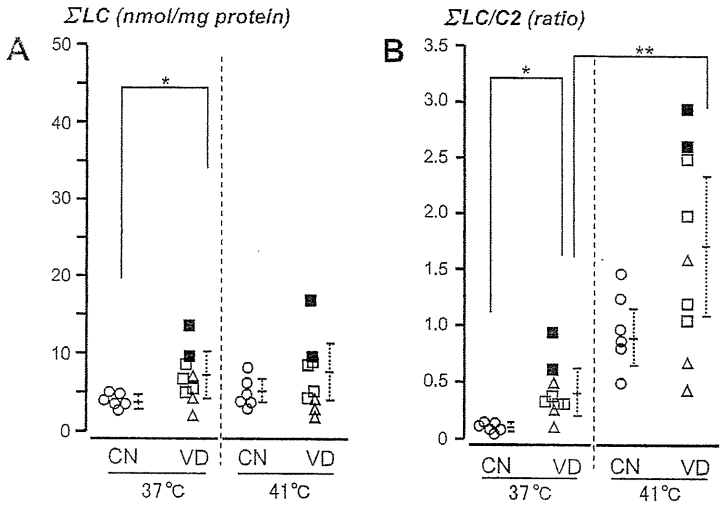

Fig. 5. Comparison of ΣLC and ΣLC/C2 of VLCAD deficiency after bezafibrate treatment. (A) ΣLC, C12 + C14 + C14:1 + C16; (B) ratio of ΣLC/C2. CN, control; VD, VLCAD deficiency; ○, Control; ■, severe VLCAD deficiency; △, intermediate form; □, myopathic form. Significant differences between different groups are shown as $^{**}p < 0.01$ and $^{*}p < 0.05$.

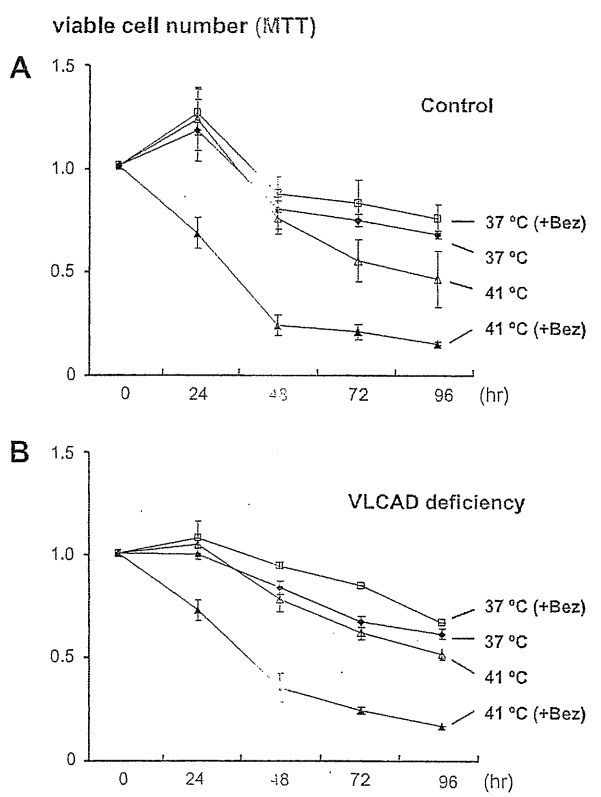

Fig. 6. Cell viability measured by MTT assay at various conditions. (A) Control; (B) VLCAD deficiency. ◆, 37 °C; △, 41 °C; □, 37 °C with bezafibrate; ▲, 41 °C with bezafibrate. Data are expressed as mean ± SD.

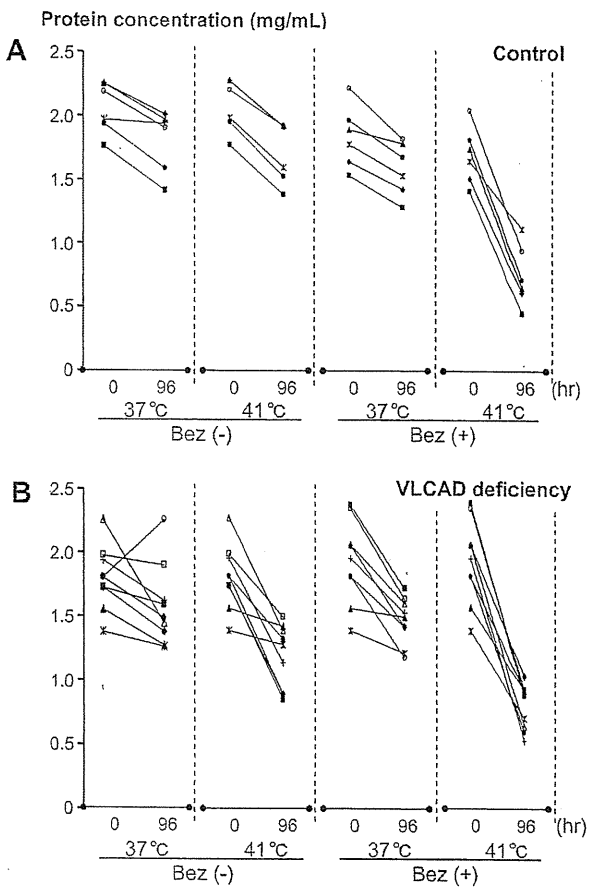

Fig. 7. Protein concentration at T_0 and T_{96} at various conditions. (A) Control; (B) VLCAD deficiency. Each line represents individual cells.

acetyl-CoA, the final product of FAO cycles, is considered to be the most important marker of the whole FAO flux, and the long-chain ACs specifically represent the long-chain FAO flux.

Our previous data indicate that in vitro AC profiling assay can identify patients with various FAO disorders [21], which is consistent with the findings of others

[22–26]. Our results showed significant reduction of the amount of C2, and accumulation of long-chain ACs in VLCAD-deficient cells. In particular, the accumulation of C12 and C14 were the most sensitive diagnostic markers to specify the clinical subgroups of VLCAD deficiency.

It has been reported that some of the milder FAO disorders are thermolabile in FAE [3,4] and that bezafibrate improves the residual VLCAD activity in patients with milder form of VLCAD deficiency [17,27]. However, the effect of heat stress or bezafibrate on FAO capacity in VLCAD deficiency by the in vitro probe assay with unlabelled palmitic acid as substrate, has never been characterized. Our approach is simpler than the other methods to determine the effects of metabolic stresses as well as of drugs on FAO disorders in vitro or in vivo.

Under heat stress, C2 significantly increased in cells from controls, intermediate/myopathic forms of VLCAD- and MCAD-deficient patients as well as in normal controls. On the other hand, C2 did not change in the severe form of VLCAD deficiency. The amounts of long-chain ACs, especially C16, were significantly elevated in all forms of VLCAD deficiency, but not in MCAD deficiency at 41 °C, suggesting that long-chain FAO are more susceptible to the heat stress, compared with medium-chain FAO. Although heat stress also increased C2 in the myopathic and intermediate forms as in normal controls, this is most likely a consequence of enhanced short- or medium-chain FAO at higher temperature (Fig. 1B). Σ[ACs] was significantly higher in the myopathic form of VLCAD deficiency compared with that of the other two forms at 41 °C, suggesting that the myopathic form of VLCAD deficiency is most sensitive to heat stress. These results are accordance with the fact that asymptomatic patients with the myopathic form often triggered the symptoms under conditions of metabolic stress [28].

The regulation of mitochondrial FAO by PPARs has been studied extensively by several groups [29]. Bezafibrate, one of the activators of PPARs, represents an alternative therapeutic approach to treat long-chain FAO disorders [17,27,30–32]. In keeping with previous studies, we also explored the effect of bezafibrate on mitochondrial FAO using our in vitro probe assay, and found that bezafibrate (0.4 mM) enhanced the long-chain FAO process. It is hypothesized that the drug diminishes the accumulation of toxic long-chain ACs in cells from VLCAD-deficient patients. We also found that bezafibrate reduced long-chain ACs more effectively in the intermediate and myopathic forms than in the severe form. These findings suggest that bezafibrate may represent a potential treatment strategy for VLCAD deficiency, specifically for the clinically milder forms.

On the other hand, all species of ACs were significantly reduced not only in VLCAD-deficient cells but also in controls, incubated with bezafibrate at 41 °C. We therefore explored the mechanism responsible for this phenomenon. Our data showed that the viable cell number significantly decreased after 24 h in the presence of bezafibrate at 41 °C. The obvious reduction of ACs is probably a consequence of loss of cell viability at this condition. These data may imply a potential toxicity of bezafibrate during fever.

In conclusion, in vitro probe acylcarnitine profiling assay using unlabelled palmitic acid as substrate is a simple and promising strategy to determine the effects of heat stress or drugs on mitochondrial FAO. Heat stress inhibits long-chain FAO specifically in long-chain FAO disorders, and bezafibrate improves impaired long-chain FAO.

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