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ESI–MS/MS study of acylcarnitine profiles in urine from patients with organic acidemias and fatty acid oxidation disorders☆

Hironori Kobayashi*, Yuki Hasegawa, Mitsuru Endo, Jamiyan Purevsuren, Seiji Yamaguchi

Department of Pediatrics, Shimane University Faculty of Medicine, 89-1 Enya, Izumo, Shimane 693-8501, Japan

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Abstract

Acylcarnitines in urine from 45 patients with organic acidemias and fatty acid oxidation disorders were evaluated using ESI–MS/MS. The urinary acylcarnitine profiles in organic acidemias, SCAD deficiency and MCAD deficiency were compatible with blood acylcarnitine profiles, and abnormalities in urinary acylcarnitine profiles in these conditions were enhanced following carnitine loading. Urinary acylcarnitine profiles were not helpful for characterization of long-chain fatty acid disorders, but a combination of urine and blood acylcarnitine analysis was useful for differential diagnosis of carnitine deficit.

Keywords: Urine; Acylcarnitine; Newborn screening; Electrospray ionization tandem mass spectrometry; Organic acidemia; Fatty acid oxidation disorder; Screening; ESI–MS/MS; Carnitine deficiency; Pivoxil; Glutaric; Aciduria type 1; GA1

1. Introduction

Mass screening of newborns for inherited metabolic disorders using electrospray ionization tandem mass spectrometry (MS/MS) is in common use worldwide; disorders including amino acidemias, urea cycle disorders, organic acidemias (OAs) and fatty acid oxidation disorders (FAODs) can be detected using this approach [1–5]. The procedure is extremely fast and sensitive compared to bioassays (Guthrie test), and only requires sampling of a blood spot. However, occasionally ambiguities arise in the analysis due to the genetic severity of the disease or the condition of the patient at the time of sample collection. In such cases, other diagnostic tools, such as urinary organic acid analysis by GC/MS, enzyme determination [6], or molecular analysis, are required for confirmation of diagnosis.

Urinary organic acid analysis using GC/MS has been used for diagnosis of OAs since the 1960s [7], and it remains an important tool for detection and evaluation of the condition of OA patients and confirmation of diagnosis following MS/MS screening. Urinary acylcarnitine analysis may also be useful in disease identification, but there have only been a few reports of MS/MS analysis of urinary acylcarnitines [8–11], even though this approach is much less time-consuming than GC/MS. We have analyzed blood acylcarnitines and urinary organic acids using MS/MS and GC/MS, respectively, in symptomatic or high-risk children for detection of inherited metabolic disorders [12], and we have also performed pilot newborn screening using MS/MS [5]. In this study, we evaluated the utility of MS/MS analysis of urinary acylcarnitines in patients with OAs, FAODs, and carnitine deficit disorders.

2. Material and methods

2.1. Subjects

Urine samples were studied from 44 patients with OAs or FAODs detected by urinary organic acid analysis, blood acylcarnitine analysis, enzyme determination, or gene analysis. The patients included 16 with methylmalonic acidemia (MMA-emia); five with propionic acidemia (PPA-emia); four each with very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency and glutaric acidemia type 2 (GA2); three with glutaric aciduria type 1 (GA1); two each with 3-methylcrotonyl-CoA carboxylase (MCC) deficiency, 3-ketothiolase (BKT) deficiency and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency;

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* Corresponding author. Tel.: +81 853 20 2219; fax: +81 853 20 2215.
E-mail address: bakki@med.shimane-u.ac.jp (H. Kobayashi).

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and one each with multiple carboxylase deficiency (MCD), 3-hydroxy-3-methylglutaryl-CoA lyase (HMGL) deficiency, short chain acyl-CoA dehydrogenase (SCAD) deficiency, tri-functional protein (TFP) deficiency, carnitine palmitoyltransferase 2 (CPT2) deficiency, and carnitine uptake defect (CUD). Three patients presenting with secondary carnitine deficiency in whom inborn metabolic disorders were suspected were also included in the study.

MMA-emia was defined as detection of both methylmalonate and methylcitrate in urine organic acid analysis. Other OAs were proven based on their characteristic urine organic acid profiles. Patients with FAODs were included if they showed typical blood acylcarnitine profiles and had a proven diagnosis using immunoenzymological methods or genetic analysis.

2.2. Sample preparation

Methanol, acetonitrile and formic acid were purchased from Wako (Osaka, Japan). The contents of an acylcarnitine (AC) reference standard kit (NSK-B, Cambridge Isotope Laboratories, Andover USA), $^{2}[H]_{9}$-carnitine, $^{2}[H]_{3}$-acetylcarnitine, $^{2}[H]_{3}$-propionylcarnitine, $^{2}[H]_{3}$-butyrylcarnitine, $^{2}[H]_{9}$-isovalerylcarnitine, $^{2}[H]_{3}$-octanoylcarnitine, $^{2}[H]_{9}$-miristoylcarnitine and $^{2}[H]_{3}$-palmitoylcarnitine, were diluted in methanol and used as an internal standard.

Acylcarnitine analysis of blood spots and serum was performed using routine methods for MS/MS [2,3]. Urine acylcarnitine analysis was performed according to the method reported by Mueller et al. [9]. Briefly, 100 μl of urine was diluted with methanol 1:10 (v/v). After a 30-min incubation, aliquots were centrifuged for 5 min at 13,000 × *g*; 10 μl of the supernatant was then transferred to a 96-well microplate and 100 μl of the methanol reference standard kit was added to each well. After drying under a gentle stream of nitrogen, 60 μl of 3N *n*-butanol–HCl was added and butylation was performed at 65 °C for 15 min. After drying, the sample was reconstituted in 100 μl of 80% acetonitrile:water (4:1 v/v).

2.3. Tandem mass spectrometry

An API 3000 triple quadrupole tandem mass spectrometer in combination with a SIL-HTc autosampler (Shimadzu, Kyoto, Japan) was used, with a sample volume of 10 μl. Quantitative analysis was achieved using ChemoView^{TM} software (Applied Biosystems/MDS SCIEX, Toronto, Canada) by comparing the signal intensity of an analyte against the corresponding internal standard. Data are expressed as mmol/mol creatinine.

3. Reference data

Reference values for urine acylcarnitines were obtained from data reported by Mueller et al. [9]. More than 30 age-matched control samples were analyzed to confirm the validity of the data (data not shown). The total ACs was represented as the sum of ACs from C3 to C18, following the approach in the reference data [9]. Reference values for newborns given by Mueller et al. were used for samples from neonates (before 1 month of age), while reference data for children of 1–5 years of age were used for infants aged over 1 month old.

4. Results

4.1. Urinary acylcarnitines in MMA-emia and PPA-emia

As shown in Table 1, 16 cases of MMA-emia (cases 1–16), and five of PPA-emia (cases 17–21) were examined. These cases included five of mild MMA-emia (cases 12–16), in which elevated excretion of relatively small amounts of methylmalonic acid and methylcitric acid were found in urinary organic acid analysis by GC/MS. Three of the 11 patients with severe MMA-emia (cases 9, 10 and 11) and two patients with mild MMA-emia (cases 15 and 16) were receiving carnitine treatment.

An increase of C3 was detected in blood acylcarnitine analysis in all but one (case 3) of the cases of severe MMA-emia. In case 3, the level of C3 in blood was only 1.44 μmol/L (cut-off, <4.0), although excretion of a large amount of methylmalonic acid and an increase in methylcitrate were found in urine. In all three patients with severe methylmalonic acidemia who were receiving carnitine treatment (cases 9–11), urinary excretion of C3 and the C3/C16 ratio were significantly enhanced, compared with patients not receiving carnitine treatment (cases 1–8). Increased excretion of C4DC, which may contain methylmalonylcarnitine, was observed in the urinary acylcarnitine profile in 10 of the 11 cases of severe MMA-emia, although the amount of C4DC varied among these cases.

No abnormalities in the blood acylcarnitine profiles were seen in the five cases of mild MMA-emia (cases 12–16), despite an increase in excretion of methylmalonic acid and methylcitric acid found in urinary organic acid analysis. Two patients with mild MMA-emia were receiving carnitine therapy (cases 15 and 16), and both showed increased excretion of C3 in urine, but neither patient had abnormalities in blood acylcarnitine analysis. In the other three cases, there were no abnormalities in acylcarnitine profiles in blood and urine.

An increase of C3 was clearly observed in urine and blood in all five cases of PPA-emia. One patient receiving carnitine therapy (case 21) showed significant enhancement of both the amount of C3 and the C3/C16 ratio.

4.2. Urinary acylcarnitines in organic acid disorders other than MMA-emia and PPA-emia

As shown in Table 2, both cases of MCC deficiency (cases 22 and 23) showed increases of C5-OH in blood acylcarnitine analysis and of methylcrotonylglycinuria in organic acid analysis. An increase in C5-OH was also observed in urine in both cases. In one case, acylcarnitine in urine was also collected after initiation of carnitine treatment (cases 22-2); in samples collected after the start of carnitine therapy the C5-OH level was enhanced significantly, and there was increased excretion of C5, C5:1 and C3, as well as total AC and C0 (Fig. 1).

MCD (case 24) was detected based on increased levels of C5-OH in blood, a characteristic profile of urinary organic acids,

Table 1
Acylcarnitines in urine and blood in MMA-emia and PPA-emia

Case no.	Age	Cr (mol/l)	Urine (mmol/mol creatinine)							Blood (μM)	
			AC	C0	C3	C5:1	C5	C4DC	C3/C16	C0	Acylcarnitines (increase)
Methylmalonic acidemia (severe)											
1	0m	2.97	24.03	4.72	7.65	1.82	0.57	2.50	26.21	7.19	C3 (5.74) C3/C2 (1.76)
2	0m	1.61	42.24	6.08	29.16	0.71	0.75	4.22	255.96	22.09	C3 (17.87) C3/C2 (1.03)
3	3m	4.32	34.64	8.97	10.03	0.28	0.65	1.93	79.65	18.71	C3 (1.44) C3/C2 (0.7)
4	10m	0.90	129.66	24.82	105.69	1.82	2.74	3.77	187.79	4.78	C3 (5.89) C3/C2(1.4)
6	2y9m	8.79	22.48	1.92	5.61	0.25	0.41	2.01	124.83	8.06	C3 (13.62) C3/C2 (2.3)
7	3y5m	1.65	52.04	9.53	1.87	2.04	0.91	26.96	3.85	18.06	C3 (5.11) C3/C2 (0.45)
8	5y4m	4.21	68.50	17.25	40.99	0.52	0.68	7.70	136.75	57.74	C3 (28.9) C3/C2 (0.71)
9*	1m	1.31	294.94	376.77	213.13	2.83	31.42	5.01	240.89	64.93	C3 (20.06) C3/C2 (0.72)
10*	2m	0.23	2691.89	1034.55	2567.10	2.63	37.38	12.72	4551.29	67.27	C3 (52.04) C3/C2 (1.21)
11*	11m	3.26	264.24	158.81	213.51	2.25	16.57	6.88	815.79	45.23	C3 (13.08) C3/C (20.67)
Methylmalonic acidemia (mild)											
12	2m	1.04	22.37	97.75	2.01	0.38	1.11	1.18	9.80	42.23	NA
13	4m	3.76	36.16	11.26	0.45	0.45	0.50	2.18	0.50	24.66	NA
14	10m	6.30	9.77	2.78	0.12	0.29	0.10	0.93	0.18	8.70	NA
15*	5m	2.62	45.81	106.39	9.00	0.63	5.20	2.35	40.16	36.66	NA
16*	3y0m	2.18	169.54	191.74	112.60	1.13	5.25	4.17	258.66	25.67	NA
Propionic acidemia											
17	1m	1.70	107.86	22.39	94.57	0.85	0.97	0.28	288.93	4.43	C3 (15.2) C3/C2 (2.59)
18	1m	0.65	13.80	3.84	7.23	0.90	0.62	0.41	38.98	7.21	C3 (18.22) C3/C2 (4.2)
19	1m	1.44	21.22	42.31	1.01	0.46	0.97	1.48	2.59	46.13	C3 (4.56) C3/C2 (0.24)
20	1y6m	3.95	10.64	5.70	5.41	0.55	0.33	0.53	88.30	22.60	C3 (17.36) C3/C2 (0.89)
21*	2y1m	1.00	872.68	11980	830.26	0.55	13.34	0.28	3429.14	12.37	C3 (35.6) C3/C2 (2.43)
Reference value											
Newborns			3.17 −45.32	1.68 −23.83	<1.86		<1.78	<2.51	<12.05	9.0 −80.0	
After 1m			3.96 −22.84	5.67 −56.09	<5.29		<0.89	<0.47	<9.00	20 −80.0	See**

* Patients under carnitine treatment; abbreviations: NA, no abnormality.
** Cut-off values of blood acylcarnitines: C3, <4.0 mM; C3/C2, <0.24. Values over cut-off are underlined.

and the clinical course. In this patient, increased excretion of C5-OH, C5:1 and C3 was seen in urine. HMGL deficiency (case 26) was detected based on increased C5-OH in blood, a characteristic profile of urinary organic acids, and the clinical course. In this case, increased excretion of C5-OH was seen in the urinary acylcarnitine profile.

Two cases of BKT deficiency (cases 26 and 27) were analyzed: one (case 27) after the start of carnitine administration. An increase in C5-OH was detected in both cases in blood, and increased urinary excretion of 2-methyl-3-hydroxybutyric acid and triglylglycine was apparent. The level of C5-OH in urine also clearly increased in both cases.

Table 2
Acylcarnitines in urine and blood in organic acid disorders other than MMA-emia and PPA-emia

Case no.	Age	Cr (mol/l)	Urine (mmol/mol creatinine)							Blood (μM)	
			AC	C0	C3	C5:1	C5	C5-OH	C5DC	C0	Acylcarnitine (increase)
3-Methylcrotonyl-CoA carboxylase deficiency											
22-1	1m	0.42	147.38	5.35	0.49	0.19	0.58	137.49	0.51	1.85	C5-OH (17.2)
22-2*	2m	0.36	601.38	156.24	11.98	13.03	37.80	505.45	0.18	53.70	C5-OH (25.9) C5:1 (0.36)
23	10m	0.66	10.25	2.79	0.24	0.22	0.25	3.09	0.04	11.46	C5-OH(2.51)
Multiple carboxylase deficiency											
24	10m	1.71	44.12	5.27	7.44	0.94	0.38	2.09	0.32	16.08	C3/C2 (0.7) C5-OH (19.34) C5:1 (0.12)
3-Hydroxy-3-methylglutaryl CoA lyase deficiency											
25	2y6m	4.19	19.90	3.75	0.18	0.34	0.16	14.17	1.33	17.48	C5-OH (2.45)
3-Methylacetoacetyl CoA thiolase deficiency											
26	7m	1.74	35.55	30.95	1.47	16.82	1.65	4.59	0.56	28.95	C5-OH(1.92) C5:1 (0.78)
27*	1y1m	0.75	35.61	76.02	0.61	13.96	1.55	6.74	0.68	44.57	C5-OH(1.3) C5:1 (0.29)
Glutaric aciduria type 1											
28-1*	7m	2.79	130.83	10.32	0.19	0.19	0.15	0.45	124.59	18.14	C5DC(1.79)
28-2*	9m	1.64	548.67	913.65	14.10	0.53	3.50	1.43	481.18	92.46	C5DC(4.8)
29	3y8m	6.83	25.28	77.88	1.58	0.95	1.34	0.95	12.29	30.11	C5DC(0.19)
30*	3y7m	5.45	174.00	458.97	11.53	1.03	2.93	0.97	119.02	27.95	C5DC(1.17)
Reference value											
	1–5 years		<22.84	5.67 −56.09	<5.29		<0.89	<0.54	<0.2	20 −80	See**

* Patients under carnitine treatment.
** Cut-off values of blood acylcarnitines: C5-OH, <1; C5:1, <0.08; C5DC, <0.15; 22-1 and 22-2 are the same cases, and 28-1 and 28-2 are also the same cases. Values over cut-off are underlined.

Four children (cases 28–31) had GA1, and samples from two (cases 30 and 31) were analyzed after the start of carnitine administration. Diagnosis of GA1 was made based on findings of increased C5DC in blood, increased excretion of glutaric acid and 3-hydroxyglutaric acid in urine, and clinical findings. Urinary excretion of C5DC was significantly elevated in all four cases, and particularly so in the two patients receiving carnitine administration.

4.3. Urinary acylcarnitines in fatty acid oxidation disorders

As shown in Table 3, urinary acylcarnitines were evaluated in 14 cases of FAODs. In the patient with SCAD deficiency (case 31), elevation of C4 was found in blood, and increased urinary excretion of ethylmalonic acid, methylsuccinic acid. In urinary acylcarnitine profile, an elevation of C4 was also observed. The two cases of MCAD deficiency (cases 32 and 33) were confirmed by their clinical courses of acute encephalopathy and sudden death, respectively. Increased excretion of suberylglycine and non-ketotic dicarboxylic aciduria was observed in the urinary organic acid profiles of these patients, and marked elevation of C8 and mild elevation of C6, C10 and C10:1 were observed in both cases.

In cases of VLCAD deficiency (cases 34–44), GA2 (cases 39–42), TFP deficiency (case 43), and CPT2 deficiency (case 44), all of which were confirmed at the enzyme and molecular levels, specific abnormalities in urinary acylcarnitine profiles were not anticipated, but characteristic acylcarnitines for each disorder were detected in blood: elevation of C14:1 in VLCAD deficiency; elevation of C4 to C14 in GA2; elevation of C16-OH, C18:1-OH in TFP deficiency; and a decrease of free carnitine and elevation of C16 in CPT2 deficiency.

4.4. Blood and urine acylcarnitines in cases with a blood carnitine deficit

Urinary acylcarnitines were investigated in four patients who presented with a clinically acute episode of encephalopathy-like symptoms and showed a free carnitine deficit in blood. As shown in Table 4, in case 45 decreased amounts of all acylcarnitines and of free carnitine were observed in blood; however, the level of free carnitine in urine was consistently high (Fig. 2A) and clearance of free carnitine was significantly higher than the control value. These findings suggest that this patient had absorption problems in the urinary tubules or a congenital carnitine uptake defect (systemic carnitine deficiency). A carnitine transport defect was confirmed in an uptake experiment using cultured fibroblasts from the patient.

In cases 46 and 47, a reduced level of free carnitine in blood was seen in MS/MS screening, whereas an increased peak for C5 was observed (Fig. 2B). The urinary acylcarnitine profiles for these two cases showed a low level of free carnitine and an

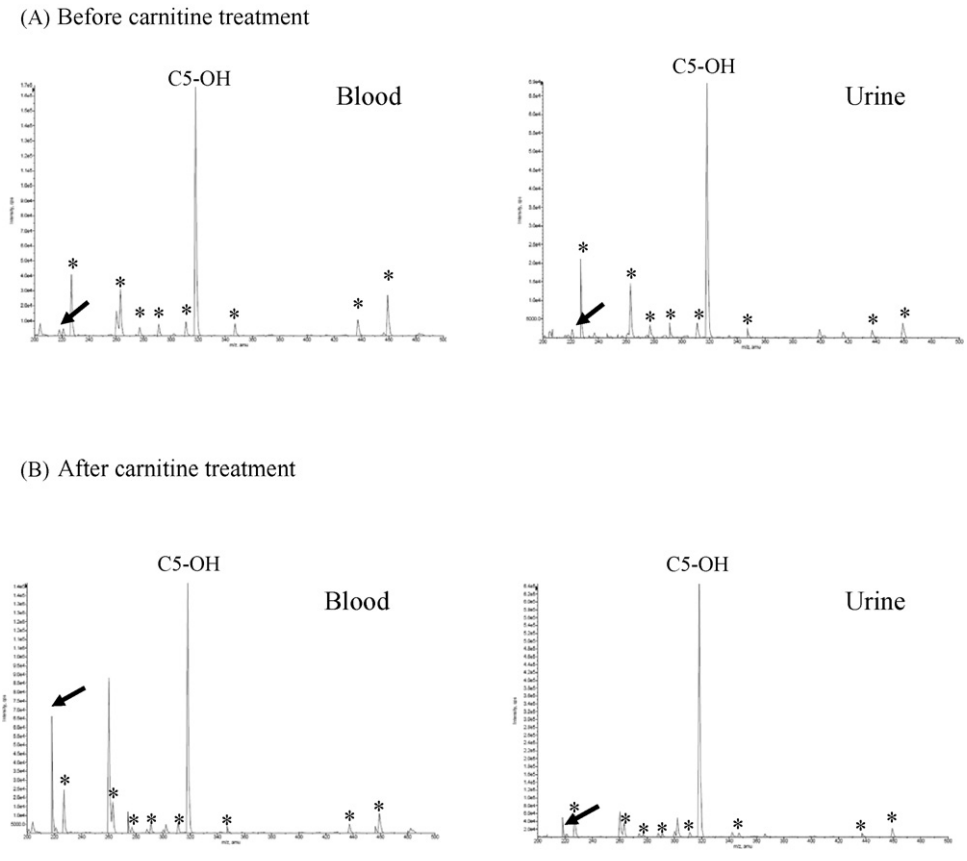

Fig. 1. Blood and urine acylcarnitine profiles in MCC deficiency (case 22). After carnitine treatment, urinary 3-hydroxyisovalerylcarnitine (C5-OH) was elevated more than blood C5-OH, based on the relative intensities of C5-OH, free carnitine (arrows) and the internal standard. (*) Peaks from the mixture of internal standards.

increased level of C5, as in the blood profiles. The clinical history of these two children showed that before sample collection they had received pivalate prodrug antibiotics for 50 of the previous 70 days and 11 of the previous 14 days, respectively. Hence, it was suggested that these two cases had secondary carnitine deficiency due to inappropriate use of antibiotics.

In case 48, a reduced level of free acylcarnitines in blood was also observed, whereas the 3-hydroxyisovalerylcarnitine (C5-OH) level was elevated. Urinary organic acid analysis showed mild elevation of methylmalonic acid and 3-hydroxypropionic acid, and elevation of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine. The urinary acylcarnitine profile also showed low free carnitine and increased C5-OH, but no elevation of C3. The clinical history of this case showed that the infant had received only special formula for over 5 months because of a severe milk allergy, suggesting that the patient had carnitine and biotin deficits due to malnutrition. The clinical condition of the patient recovered soon after a change to milk containing carnitine and biotin.

5. Discussion

Acylcarnitine analysis using MS/MS and blood filter paper is increasingly common in newborn mass screening [1–5] and diagnosis of inherited metabolic disorders. MS/MS is a very sensitive, fast and powerful tool, and acylcarnitine screening by MS/MS requires only a small piece of filter paper containing dried blood. However, it is sometimes difficult to make a definite diagnosis by MS/MS analysis alone [6,13], and a confirmatory test such as urinary organic acid analysis using GC/MS [12], enzyme determination, or molecular analysis may be required [3]. Urinary organic acid analysis is useful for confirmation of diagnosis of many kinds of OAs, and often provides more detailed information on the condition and severity of OA patients; however, this technique is time-consuming and requires special skills for interpretation of data [12,14].

In the current study, we examined the utility of urinary acylcarnitine analysis for diagnosis and evaluation of disorders detected in screening. The advantages of this approach are a short analysis time and high sensitivity, with only a very small amount of urine required. Furthermore, comparison of metabolic profiles between blood and urine and between acylcarnitines and organic acids may be informative for confirmation of diagnosis and understanding of pathophysiology. In performing this analysis, it is important to recognize that urinary levels of metabolites can be variable under different renal conditions. Furthermore, Mueller et al. reported that the within-run and run-to-run CVs for acylcarnitine levels (per unit of creatinine) were between 5.2% and 23.2% and between 7.0% and 38.2%, respectively [9]. However, we obtained consistent values over 30 controls

Table 3
Acylcarnitines in urine and blood in fatty acid oxidation disorders

Case no.	Age	Cr (mol/l)	Urine (mmol/mol creatinine)										Blood	
			C0	C4	C5DC	C6	C8	C10	C14:1	C14	C16	C16:1-OH	C0	ACs (increase)
SCAD deficiency														
31	1m	0.67	21.63	5.48	1.64	0.80	0.71	0.16	0.26	0.53	0.32	0.06	55.12	C4 (6.32)
MCAD deficiency														
32	1y7m	6.28	6.07	1.50	0.65	1.42	26.42	0.74	0.07	0.42	0.11	0.02	23.27	C6(1.57) C8 (4.75) C10 (0.58) C10:1 (0.41)
33	6y0m	3.27	57.32	3.14	1.08	2.36	41.48	0.98	0.22	0.71	0.13	0.04	12.46	C6 (0.36) C8(1.57)
VLCAD deficiency														
34	3m	0.37	12.38	1.14	1.38	0.08	0.39	0.43	0.40	1.13	2.13	0.00	2.74	14:1(0.7)
35*	3m	2.51	174.09	0.93	1.17	0.46	1.72	0.31	0.27	3.67	1.42	0.10	17.36	C14:1 (4.63) C14 (2.52)
36	3m	0.57	8.47	2.16	4.46	0.61	0.34	0.29	0.21	0.27	0.17	0.07	69.40	C14:1 (4.5) C14 (2.22)
37*	3m	2.55	1475.5	5.85	2.68	2.13	9.80	0.48	0.92	1.81	0.83	0.08	36.26	C14:1 (0.47)[a]
38*	1y5m	0.62	175.41	4.70	1.08	0.84	0.96	0.21	0.20	0.25	0.26	0.11	33.10	C14:1 (0.82) C14 (1.18)
Glutaric aciduria type 2														
39	0m	0.51	17.07	31.03	11.27	1.89	2.32	0.93	0.16	0.16	0.00	0.00	6.62	C4 (3.99)[a] C8 (5.05) C10 (1.01) C14:1 (1.56)
40*	0m	1.75	92.23	4.14	6.13	0.22	0.71	0.10	0.21	0.81	0.18	0.12	56.06	C6 (1.8)[a] C8(1.98) C10 (1.67) C14:1 (1.33)
41*	1m	2.40	76.86	2.20	0.57	0.12	0.64	0.24	0.14	0.31	0.08	0.06	48.91	C14:1 (0.55)
42	12y	8.32	3.18	1.03	3.80	0.07	0.12	0.06	0.03	0.0	0.08	0.01	38.13	NA
TFP deficiency														
43	1m	3.34	8.25	0.76	0.88	0.14	1.13	0.25	0.31	2.97	0.78	0.17	19.26	C16OH(1.57) C18:1OH(0.76)
CPT2 deficiency														
44	5m	2.51	2.03	0.24	0.61	0.07	0.13	0.19	0.20	0.14	0.30	0.06	1.01	C16 (2.80)[a]
Reference value														
Newborns			1.65 −23.83	<4.08	<2.25	<1.14	<0.85	<0.35	<2.56	<0.58	<0.44	<1.46	9.0 −80.0	
After 1m			5.67 −56.09	<0.74	<0.2	<0.48	<0.39	<0.36	<0.23	<0.51	<1.55	<0.16	20.0 −80.0	See**

* Patients under carnitine treatment. NA, no abnormality; a, serum samples.
** Cut-off values of blood acylcarnitines: blood paper: C4, <1.2; C6, <0.25; C8, <0.35; C10, 0.30; C10:1, <0.20; C14, <0.7; C14:1, <0.42; C16-OH, <0.10; C18:1-OH, <0.07; C16, <1.0. Values over cut-off are underlined.

samples, and creatinine-related reference values were used in the study with only minor changes.

MMA-emia and PPA-emia are the most common OAs, and are detectable through an elevation of C3, C3/C2 or C3/C16 in MS/MS screening using blood filter paper. However, diagnostic difficulty using MS/MS screening of dried blood spots has been reported in some cases of MMA (particularly for the mild form), and in such cases a confirmatory diagnosis can be made by GC/MS analysis of urine samples [5,13]. Our results also suggested that the mild form of MMA cannot be detected in MS/MS screening, although some cases might be detectable with urinary acylcarnitine analysis under carnitine loading. Of five cases with propionic acidemia, only one, which was asymptomatic and detected in newborn mass screening, showed no abnormalities in the urinary acylcarnitine profile, although elevation of diagnostic markers was seen. In the other case detected in newborn mass screening, a case of MCC deficiency, elevation of marker acylcarnitines was clearly apparent in both urine and blood in the asymptomatic period. Cases of GA1 have been reported in which C5DC (glutarylcarnitine) and urinary excretion of glutaric acid are low [15], and Tortorelli et al. [8] reported that urinary acylcarnitine analysis is an informative tool in bio-

Table 4
Blood and urine acylcarnitines in cases with a blood carnitine deficit

Case	Age	Cr (mol/l)	Urine (mmol/mol creatinine)					Blood (nM)		Carnitine clearance (%)
			AC	C0	C2	C5	C5-OH	C0	Acyl carnitine (increase)	
Carnitine transporter defect										
45	2y0m	1.21	6.34	22.15	29.27	0.27	0.22	8.00		4.30
Secondary carnitine deficiency antibiotics (cephem pivoxil)										
46-1	2y1m	3.32	22.41	0.79	0.53	21.17	0.08	0.73	C5(1.12)	1.40
46-2	2y2m	2.58	2.45	1.58	0.97	0.12	0.11	9.78	C5(0.13)	0.30
47	4y2m	3.72	82.59	0.53	0.81	80.83	0.18	0.50	C5(2.13)	0.12
malnutrition (severe milk allergy)										
48	5m	5.36	10.01	1.72	0.75	0.08	6.81	8.59	C5-OH(3.72)	0.10
Reference value										
1–5 years			<22.84	5.67 −56.09	<60.48	<0.89	<0.54	20 −80	See*	2.00

* Reference values of blood acylcarnitines: C5-OH, <1; C5, <1.0 42-1 and 42-2 are the same case. Values over cut-off are underlined.

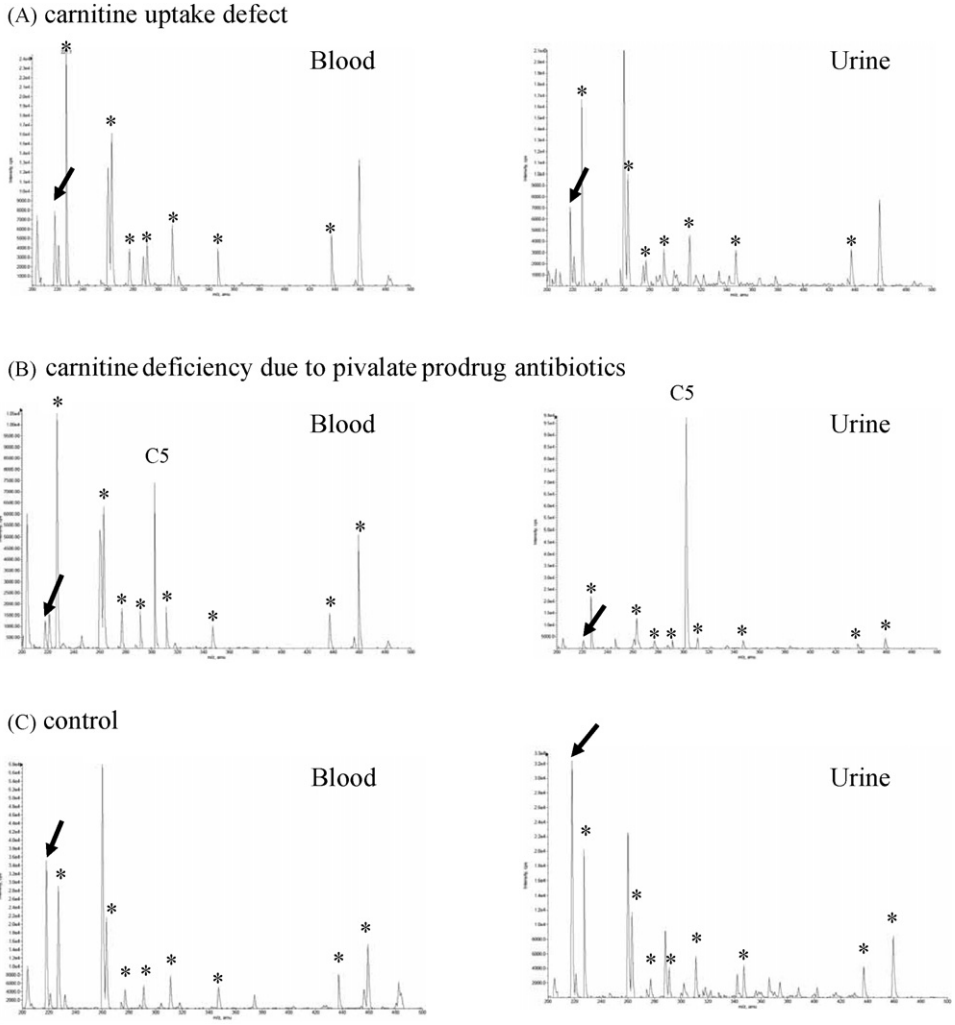

Fig. 2. Blood and urine acylcarnitine profiles in carnitine deficiency (cases 45 and 46). Patient with CUD (A) excreted a large amount of free carnitine in urine (arrows) compared to free carnitine in blood. Following treatment with pivalate prodrug antibiotics (B), patients with secondary carnitine deficiency showed a large peak for pivaloylcarnitine in both blood and urine, whereas only a very small amount of free carnitine was present in urine. (C) Control data from a 2-year-old child. (*) Peaks from the mixture of internal standards.

chemical diagnosis of GA1. Increased excretion of C5DC in urine was observed in at least three cases in the current study.

Diagnostic acylcarnitine markers for each OA showed an increase with carnitine loading. Even in the mild form of MMA-emia, the levels of C3 and C3/C16 increased in urine after carnitine loading, despite the absence of abnormalities in blood. Therefore, in cases of OAs with ambiguous metabolic profiles or borderline marker levels, urinary acylcarnitine analysis may be helpful for confirmation of diagnosis, particularly after carnitine loading. In FAODs, urinary acylcarnitine profiles in SCAD deficiency and MCAD deficiency were similar to blood acylcarnitine profiles. Additionally, it appears unlikely that urinary acylcarnitine analysis will be useful for cases of long-chain fatty acid disorders including VLCAD deficiency, TFP deficiency or CPT2 deficiency.

In MS/MS screening, a free carnitine deficit was found in four patients who presented clinically with an acute encephalopathy-like illness. In such cases, urinary acylcarnitine analysis was helpful for differential diagnosis of disorders. Carnitine treatment is important in cases with systemic carnitine deficiency, whereas administration of drugs [15,16] or nutritional approaches should be reconsidered in other cases. MS/MS screening of samples from newborns has the potential to detect OAs, FAODs and amino acid disorders in pre-symptomatic stage, and early diagnosis and intervention is essential for a favorable outcome in such children. Precise diagnosis of disease or clinical types is also required, and other diagnostic tools, including urinary organic acid analysis, enzyme determination, or molecular analysis, should also be part of the screening system. Our results suggest that urinary acylcarnitine analysis is also useful for evaluation of some OAs and FAODs, and further investigation of urinary acylcarnitines in more cases and other disorders will determine the significance of this approach. Latest manuscripts [17,18] promote measurement of acylcarnitines without derivatization. Current method remains potential to develop simpler method by non-derivatized method. Further investigation will also be needed.

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