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Selective screening for fatty acid oxidation disorders by tandem mass spectrometry: difficulties in practical discrimination

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Abstract

In a selective screening for fatty acid oxidation disorders by tandem mass spectrometry, we tested the diagnostic ratios and acylcarnitine concentrations in sera or blood spots, which were reported to be specific to very long-chain acyl CoA dehydrogenase deficiency, carnitine palmitoyltransferase I deficiency, and carnitine palmitoyltransferase II deficiency. While the acylcarnitine profiles in the majority of these patients were typical in the respective disorders, some overlapping of the indices was observed between these patients and the infants, who showed symptoms mainly related to hypoglycemia but did not have the disorders mentioned above. Although the diagnostic ratio of tetradecenoylcarnitine to dodecanoylcarnitine for very long-chain acyl CoA dehydrogenase deficiency seemed to minimize the overlapping in this study, additional measures including careful assessment of clinical data and enzyme assays may be necessary for the diagnosis in atypical cases.

Keywords: Fatty acid oxidation disorders; Fatty acids

1. Introduction

Since the initial application of tandem mass spectrometry to acylcarnitine analysis for the screening of inborn errors of metabolism [1,2], electrospray tandem mass spectrometry (ESI-MS–MS) has been successfully applied in newborn screening and selective screening for organic acidemias and fatty acid oxidation disorders [3–7]. Although the diagnosis of these disorders should be confirmed by enzyme assay and/or DNA analysis, the ESI-MS–MS measurement is less laborious and provides a rapid and accurate diagnosis, characteristics that are important for a favorable patient outcome [8]. In earlier reports, the diagnosis of these disorders depended on the profiling of acylcarnitines in blood [9]. In newborn screening, however, a quantitative analysis of acylcarnitines is essential to screen patients with the

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above-mentioned disorders, and various diagnostic ratios, such as the ratio of propionylcarnitine to acetylcarnitine for propionic acidemia and methylmalonic acidemia, have been tested [7,8]. In the selective screening of very long-chain acyl CoA dehydrogenase (VLCAD) deficiency, the patients could be distinguished from the controls by referring to the serum concentrations of tetradecenoylcarnitine (C14:1-carnitine) and the ratio of C14:1-carnitine to C8:1-carnitine [10]. Then, it was reported that the ratio of free carnitine to the sum of C16-carnitine and C18-carnitine (C0/(C16+C18)) in blood spots were highly specific for carnitine palmitoyltransferase (CPT) I deficiency [11], and that the ratio of the sum of C16-carnitine and C18:1-carnitine to acetylcarnitine ((C16+C18:1)/C2) in sera detected all CPT II deficiencies [12].

We tested the reported diagnostic ratios and acylcarnitine concentrations in sera or blood spots in our selective screening of the above-mentioned disorders, together with the additional diagnostic ratios.

2. Experimental

2.1. Materials

Stable isotope-labelled acylcarnitines used in this study were synthesized in our laboratories [13], except for L-[$^{2}H_{3}$]hexadecanoylcarnitine, which was purchased from the VU Medical Center Metabolic Laboratory, Amsterdam, The Netherlands.

2.2. Samples

In our selective screening, clinical and laboratory findings of patients were characterized by hypoglycemia, hyperammonemia, elevated creatinine phosphokinase (CK) levels, lactic acidemia, muscle weakness, consciousness disturbance, convulsions, sudden death, or acute life-threatening events in infancy. Blood samples were collected by venepuncture during or after the acute events, and frozen serum or plasma was transferred to our laboratories and stored in a freezer until analysis. In some patients, for the diagnosis of CPT I deficiency, blood spots were also prepared using the same filter papers as those used in general newborn screening in Japan. Fifty four reference serum samples were obtained from hospitalized children aged 2 months–14 years, and none of them showed the findings mentioned above.

Twelve patients with VLCAD deficiency aged 3–29 years showed typical muscle weakness and high CK levels; a patient aged 13 years had experienced a hypoglycemic crisis at the age of 5 months. The diagnosis of these patients was confirmed by enzyme assay [14,15]. Six children aged 2–7 years who experienced a hypoglycemic attack with or without marked ketosis and one child aged 2 years who had episodes of high CK values were investigated, since they had serum C14:1-carnitine levels higher than 0.5 nmol/ml and normal VLCAD activities.

Four patients with CPT I deficiency aged 1 month–5 years showed multiple episodes of hypoglycemic crisis. The diagnosis of these patients was confirmed by enzyme assay according to the reported methods [16,17]. An infant, who had experienced severe asphyxia with profound hypoglycemia ($<$0.5 mM) shortly after birth, was investigated because of a low C16-carnitine level in a blood spot, which was collected at the age of 20 days for newborn screening. She has not had any additional hypoglycemic attack without specific therapies, and the C16- and C18-carnitine levels in her blood spots were maintained within the reference ranges in her later life, although she currently has marked brain atrophy and severe psychomotor retardation.

Five patients with CPT II aged 1–24 years had muscle weakness and high CK levels, or hypoglycemic attacks, and the diagnosis was confirmed by enzyme assay [16,17]. An infant who had experienced hypoglycemia in his first day of life was investigated because of a high serum C16-carnitine level; the diagnosis of glycogen storage disease type Ib was confirmed enzymatically. Another infant who had had a hypoglycemic attack at the age of 4 days was investigated because of a high serum C16-carnitine level. Although the diagnosis of this patient has not been established because of the lack of consent for further investigation, she has not experienced an additional hypoglycemic crisis and has been doing well without muscle symptoms for 18 months.

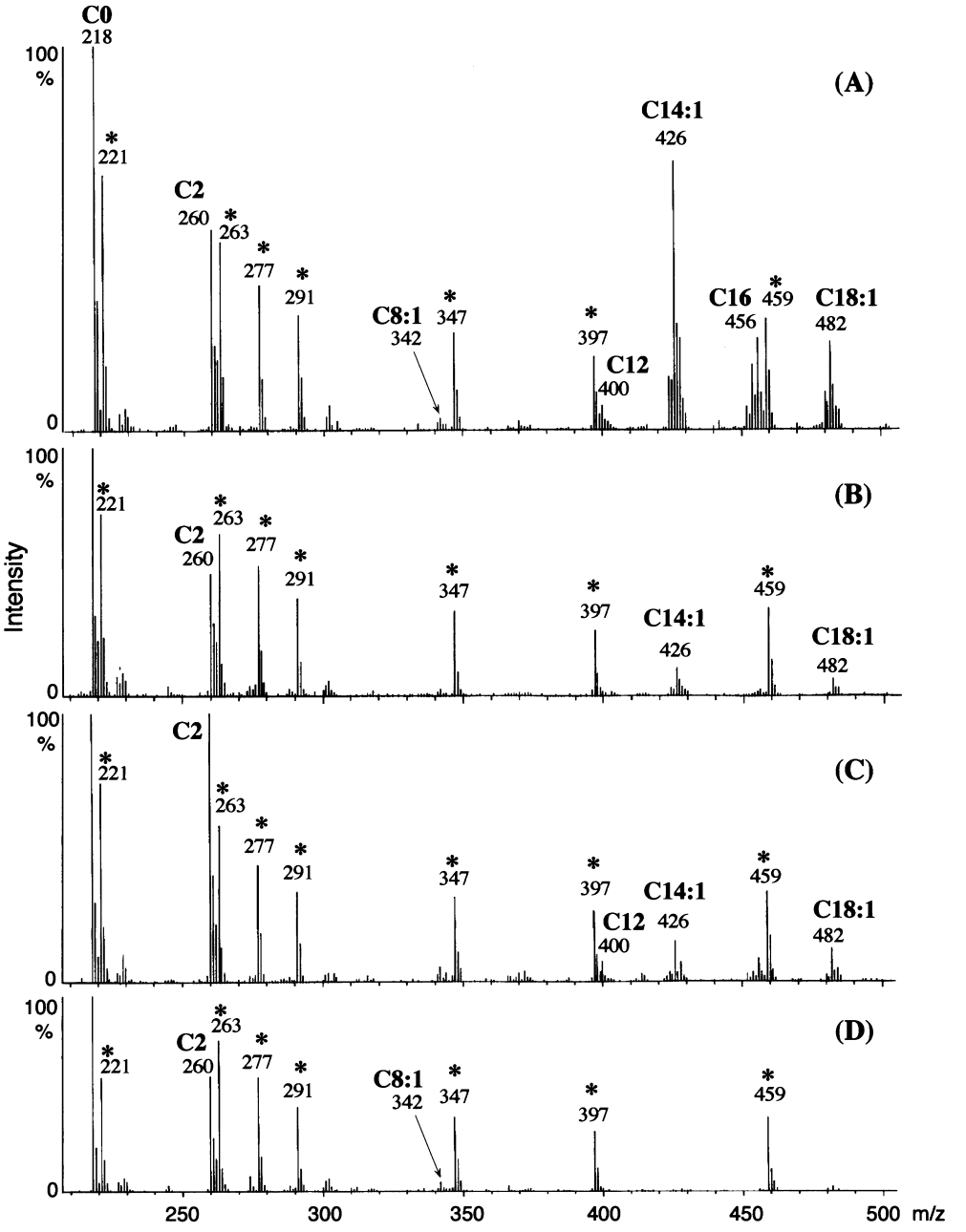

Fig. 1. Serum acylcarnitine profiles obtained by ESI-MS–MS analysis with precursor ion scanning of m/z 85. (A) A patient with VLCAD deficiency, presenting muscle weakness during a febrile illness at the age of 3 years. (B) A patient with VLCAD deficiency, presenting rhabdomyolysis and respiratory failure during a febrile illness at the age of 4 years. (C) A patient aged 4 years, presenting several episodes of ketotic hypoglycemia; VLCAD activities in cultured skin fibroblasts were normal, and urinary organic acid profile was atypical for glutaric aciduria type II. (D) A control infant. The ion peaks indicate the molecular ions of the butyrated acylcarnitines. Their masses are as follows: free carnitine (C0, 218), acetyl (C2, 260), C8:1-acyl (C8:1, 342), C10-acyl (C10, 372), C12-acyl (C12, 400), C14:1-acyl (C14:1, 426), C16-acyl (C16, 456), C18:1-acyl (C18:1, 482). The ion peaks highlighted with an asterisk indicate the stable isotope-labeled internal standards. Their masses are as follows: free carnitine-$^2H_3$ (221), acetylcarnitine-$^2H_3$ (263), propionylcarnitine-$^2H_3$ (277), butyrylcarnitine-$^2H_3$ (291), octanoylcarnitine-$^2H_3$ (347), glutarylcarnitine-$^2H_9$ (397), and palmitoylcarnitine-$^2H_3$ (459).

2.3. Sample preparation and mass spectrometry

In the analysis of blood spots, a microplate sample process was carried out using the previously reported methods [7,18]. In the analysis of serum or plasma samples, the mixture of 18 μl of the sample and 660 μl of the same methanol solution containing stable isotope-labeled standards as that used for blood spot analysis was centrifuged at 10 000 *g* for 10 min. The supernatant was dried under a nitrogen stream, and the dry residue was derivatized with buthanolic HCl. The sample was dried again under a nitrogen stream, redissolved in 120 μl of 50% acetonitrile, and transferred into a microplate well.

ESI-MS–MS analysis was done using a model TSQ7000 triple-stage mass spectrometer (ThermoQuest, Tokyo, Japan) equipped with a model LC10 HPLC system and a model SIL-10ADVP autoinjector (Shimadzu, Kyoto, Japan) [7].

The concentrations of C12-carnitine were calculated using octanoylcarnitine-$^2H_3$ as an internal standard, and those of C14:1-, C16-, C18- and C18:1-carnitines using hexadecanoylcarnitine-$^2H_3$ or hexadecanoylcarnitine-$^2H_9$.

The day-to-day coefficients of variation (10 measurements in 2 weeks) in blood spot of a control subject were 12.1% for free carnitine, 7.7% for C16-carnitine and 13.2% for C18-carnitine, while those in serum of a patient with VLCAD deficiency were 10.7% for free carnitine, 14.6% for acetylcarnitine, 17.8% for C8:1-carnitine, 15.1% for C12-carnitine, 9.2% for C14:1-carnitine, 5.8% for C16-carnitine, and 9.4% for C18:1-carnitine.

3. Results

The precursor ion mass spectra of sera of VLCAD-deficient patients with high and relatively low C14:1-carnitine levels, together with that of a child with hypoglycemia, are shown in Fig. 1. The serum concentrations of C14:1-carnitine in patients with

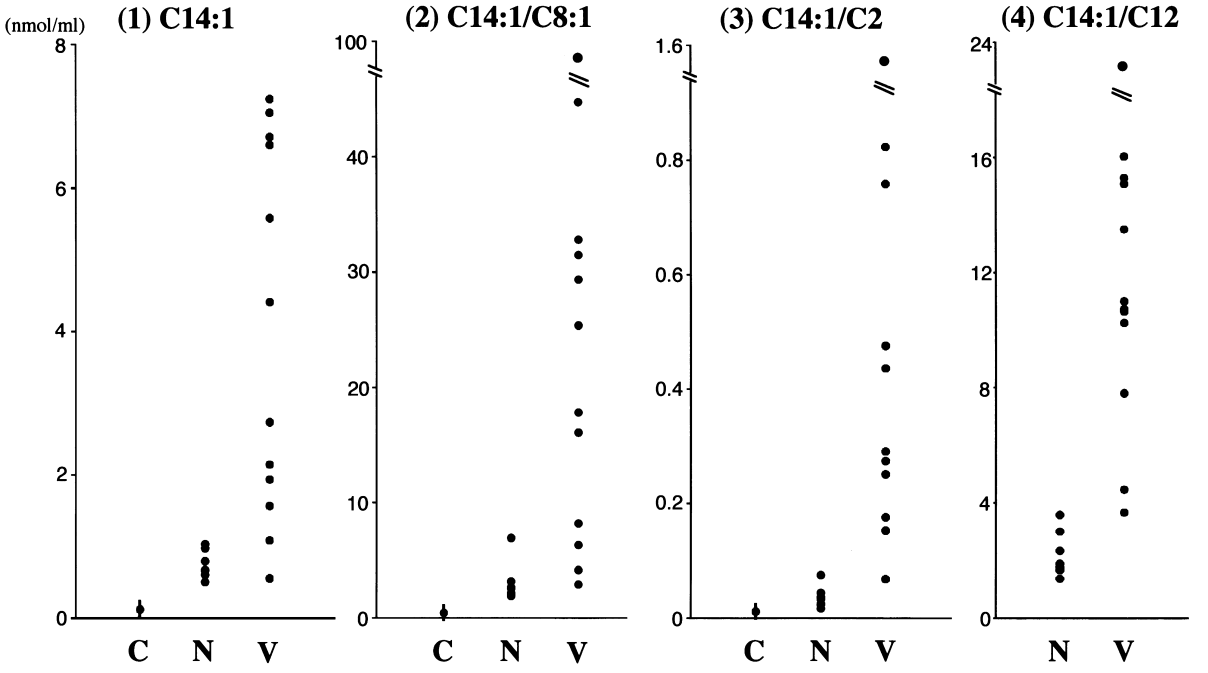

Fig. 2. The diagnostic acylcarnitine concentrations and ratios in sera for VLCAD deficiency in controls (C), non-VLCAD-deficient infants with higher C14:1-carnitine concentrations higher than 0.5 nmol/ml (N), and VLCAD-deficient patients (V). The abbreviations are: C14:1, C14:1-carnitine; C8:1, C8:1-carnitine; C2, acetylcarnitine; C12, C12-carnitine.

VLCAD deficiency ranged from 0.55 to 7.24 nmol/ml, which were higher than the 99th percentile of reference range (0.32 nmol/ml), while those in the children with high C14:1-carnitine levels but normal VLCAD activities ranged from 0.5 to 1.03 nmol/ml (Fig. 2). Eight VLCAD-deficient patients had C14:1-carnitine levels more than 2.0 nmol/ml and the acylcarnitine profile was specific to this disorder. Among the VLCAD-deficient patients and hypoglycemic children, the overlap of the ratios of C14:1-carnitine to acetylcarnitine or to C12-carnitine was less apparent than that of C14:1-carnitine concentrations.

The precursor ion mass spectra of blood spots of a patient with CPT I deficiency and a child with asphyxia are shown in Fig. 3. Free carnitine levels and the ratio C0/(C16+C18) in blood spots of four patients with CPT I deficiency were markedly higher than the 99th percentile of reference range in newborn blood spots (55.6 nmol/ml and 18.4, respectively) (Fig. 4). The ratio of the infant with asphyxia was 151 and equivalent to the reported values in patients with CPT I deficiency.

The precursor ion mass spectra of blood spots of patients with CPT II deficiency and a patient with glycogen storage disease type 1b are shown in Fig. 5. The mass spectrum of one patient with CPT II deficiency was featured by mildly elevated C16- and C18:1-carnitines, and that of the other patient by elevated C8- and C10-dioylcarnitine in addition to elevated C16- and C18:1-carnitines. The combined levels of C16-carnitine and C18:1-carnitine and the ratios (C16+C18:1)/C2 in sera of the patients with CPT II deficiency were markedly higher than the 99th percentile of reference range (0.45 nmol/ml and 0.028, respectively). Those of a patient with glycogen storage disease and an infant with an episode of neonatal hypoglycemia were distributed in a range of the patients with CPT II deficiency (Fig. 6).

4. Discussion

In this study, we showed that, using the diagnostic acylcarnitine(s) levels or the designated ratios, the patients with VLCAD deficiency, CPT I deficiency, or CPT II deficiency could not be clearly distinguished from the selected children with such symptoms as hypoglycemia and that the ratios of C14:1-carnitine to C12-carnitine reduced the overlap in the screening of VLCAD deficiency.

Since confirmatory assays in fatty acid oxidation defects in mitochondria and a group of organic acidemias may be laborious and require special expertise, the analysis of the metabolites related to these disorders were conducted [19], and acylcarnitine analysis by tandem mass spectrometry has been shown to be a powerful selective screening tool for these disorders [10]. In the early investigations of acylcarnitines in patients with these disorders, typical acylcarnitine profiles were reported [1,9,20–23]. Indeed, the acylcarnitine profiles were very specific to the respective diseases in the majority of the patients identified in this study, while urinary organic acid analysis by gas chromatography–mass spectrometry gave non-specific results, including abnormal excretion of dicarboxylic acids or negative results. However, we sometimes encountered atypical results in our routine practice of acylcarnitine analysis, such as a mild increase of C14:1-carnitine in serum samples.

In newborn screening, on the other hand, research on the analytical precision, cutoffs and confounding factors affecting the interpretation of quantitative data has been conducted [4,24]. In the newborn or selective screening of VLCAD deficiency, the C14:1-carnitine or C14:2-carnitine concentration [4,25], the ratio of C14:1-carnitine to C8:1-carnitine [10], and the ratio of C14:1-carnitine to C12:1-carnitine [26] have been reported to be diagnostic. We used the ratio of C14:1-carnitine to C12-carnitine, instead of C12:1-carnitine, because of the interference of labeled glutarylcarnitine as an internal standard on C12:1-carnitine, and showed that this ratio may be more diagnostic than the C14:1-carnitine level for VLCAD deficiency. In this study, the ratio of C14:1-carnitine to acetylcarnitine was also assumed to be diagnostic, which suggested that the increased amounts of medium- and long-chain acylcarnitines, together with acetylcarnitine, appeared in the blood of young subjects without VLCAD deficiency but with conditions such as hypoglycemia. However, these two ratios were in-

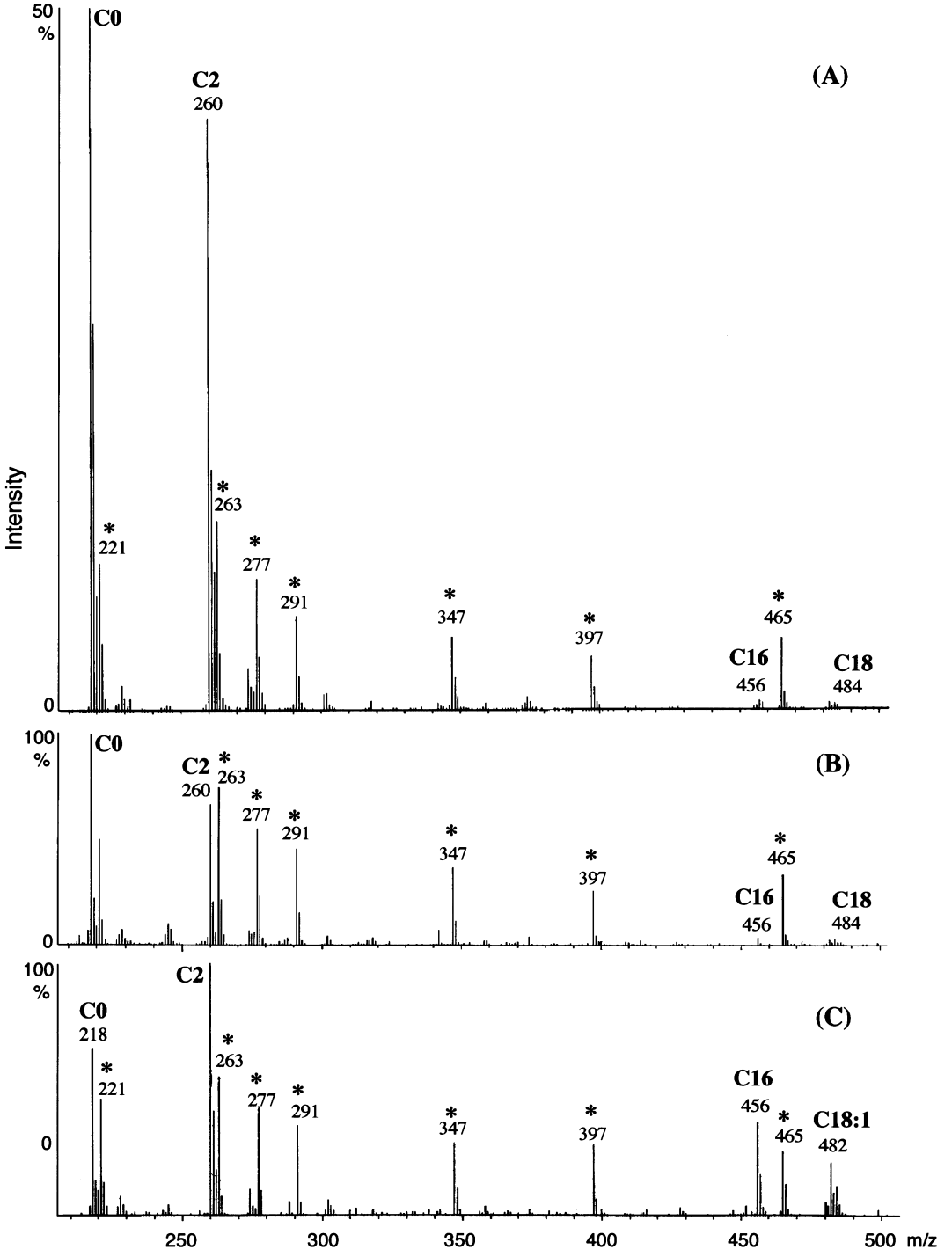

Fig. 3. Blood spot acylcarnitine profiles obtained by ESI-MS–MS analysis with precursor ion scanning of m/z 85. (A) A patient with CPT I deficiency, presenting failure-to-thrive, developmental delay, a hypoglycemic crisis and sudden death at the age of 15 months. (B) An infant who experienced severe asphyxia with profound hypoglycemia (see the text for additional information). (C) A control infant. See Fig. 1 about the masses of butyrated acylcarnitines, except for hexadecanoylcarnitine-$^{2}H_{9}$ (*, 465).

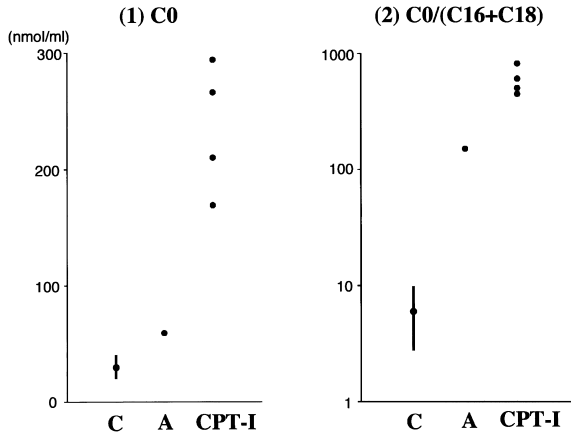

Fig. 4. The diagnostic acylcarnitine concentrations and ratios in sera for CPT I deficiency in controls (C), an infant with severe asphyxia (A), and CPT I deficient patients (CPT-I). The abbreviations are: C0, free carnitine; C16, C16-carnitine; C18, C18-carnitine.

sufficient for clearly distinguishing VLCAD deficiency from the other conditions.

It is noteworthy that, in the diagnosis of MCAD deficiency, mildly elevated C8-carnitine values in blood have been reported in several patients with myopathies, including mitochondrial respiratory-chain disorder and argininosuccinic aciduria, and that these conditions were successfully distinguished from MCAD deficiency using the ratio of C8-carnitine to C10-carnitine [27]. In this report, interestingly, C8-carnitine levels in blood of asymptomatic patients with MCAD deficiency were somewhat higher than those of symptomatic patients. In our study, a patient with VLCAD deficiency who had experienced multiple crises of rhabdomyolysis showed a relatively low serum level of C14:1-carnitine.

For the diagnosis of CPT I deficiency, the ratio C0/(C16+C18) in blood spots was reported to be sensitive in newborn or selective screening [11]. In this report, 0.04% of newborns had levels of C16- and C18-carnitine that were lower than the 0.1th percentile in newborn screening, while only the patients with CPT-I deficiency exceeded the cut-off value of 100 for C0/(C16+C18). In addition, interestingly, this diagnostic ratio in the infants more than 15-days-old was markedly higher than those in younger newborns. Thus, in our study, the sampling date of 20 day and the special condition of severe hypoglycemia may contribute to a high C0/(C16+C18) ratio of an asphyxic infant, although the mechanism for the low levels of long-chain acylcarnitines in a blood spot is not clear.

Gempel et al. reported that the ratio $(C16+C_{18})/C2$ in sera detected all CPT II deficiencies and distinguished them from unspecific alterations of serum acylcarnitines [12,28]. In their study, they suggested that the elevations of serum acylcarnitines were due to an unspecific release from necrotic muscle fibers of symptomatic subjects. In our study, most of the samples were collected when the subjects were symptomatic, and the ratio (C16+C18)/C2 did not add any other sensitive information for screening for the CPT-II deficiency than the sum of C16- and C18:1-carnitine. High values of long-chain acylcarnitines in the two subjects without CPT-II deficiency could be due to an unspecific release from some necrotic cells, and the additional analysis of the samples obtained during asymptomatic period may be a choice for confirmation of this disorder. As shown in Fig. 3, C8- and C10-dioylcarnitines, together with long-chain acylcarnitines, were elevated in a patient with CPT-II deficiency as well as in a patient with a severe form of carnitine/acylcarnitine translocase deficiency [29], and the latter disorder is expected to be distinguished from the other by enzyme assays.

Careful evaluation of the clinical data such as CK and uric acid values of the patients appears to be important [30]. However, these values, except for blood sugar, were rarely measured in an emergency, and hypoglycemia was the only condition considered in the selective screening of the majority of cases. In our case with glycogen storage disease Ib, careful evaluation of the clinical data, including persistent lactic acidemia together with hypoglycemia, provided the proper clue for the diagnosis. Another interesting patient with glutaric aciduria type II, who first presented at 4 days with severe metabolic acidosis, did not show the typical acylcarnitine profile in a blood spot at that time, and the typical profile was demonstrated at 12 days after carnitine infusion [31]. These cases, including the patient

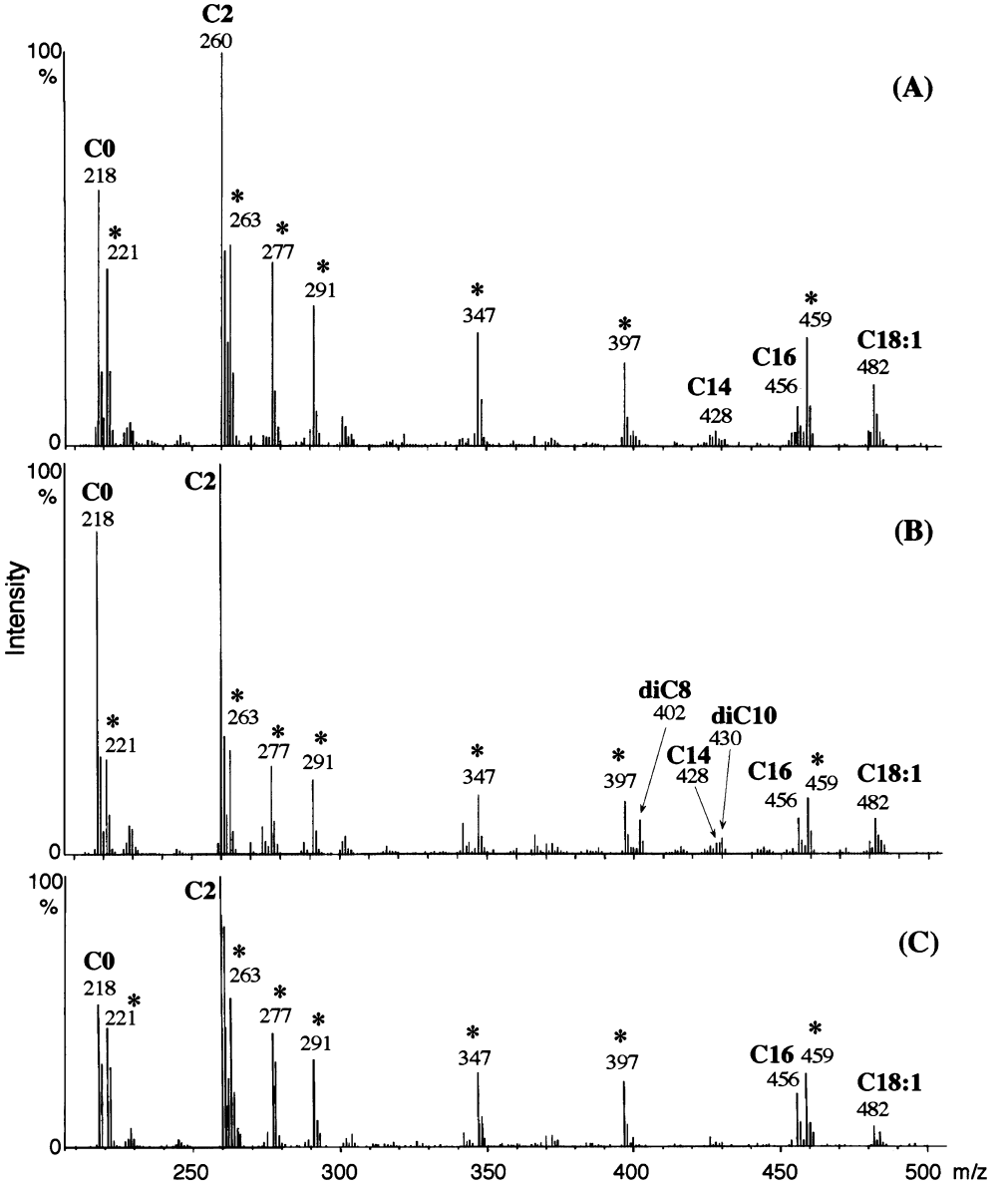

Fig. 5. Serum acylcarnitine profiles obtained by ESI-MS–MS analysis with precursor ion scanning of m/z 85. (A) A patient with CPT II deficiency, presenting multiple episodes of hypoglycemia and hepatomegaly at the age of 3 years. (B) A patient with CPT II deficiency, presenting the first episode of muscle pain and high serum CK values at the age of 22 years. (C) An infant with glycogen storage disease type Ib, presenting hypoglycemia in his first day of life. See Fig. 1 about the masses of butyrated acylcarnitines, except for C8-dioylcanritine (diC8, 402), C10-dioylcanritine (diC10, 428).

reported by Gempel et al., suggest the importance of the analysis of multiple or serial samples by ESI-MS–MS, which is less laborious than the other confirmatory tests for fatty acid oxidation disorders.

5. Conclusion

The present data indicate that there is overlap between patients with fatty acid oxidation disorders

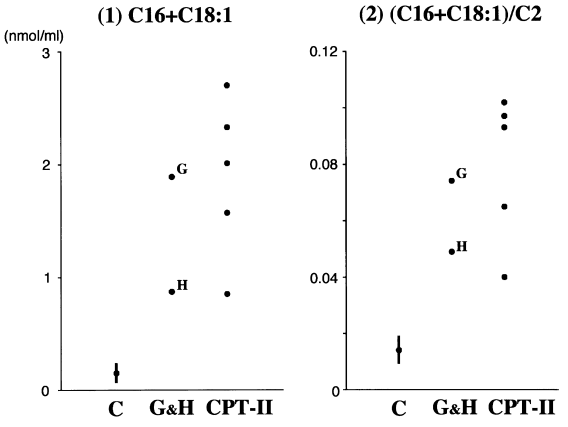

Fig. 6. The diagnostic acylcarnitine concentrations and ratios in sera for CPT II deficiency in controls (C), a patient with glycogen storage disease type Ib (G), an infant with neonatal hypoglycemia (H), and CPT II-deficient patients (CPT-II); see the text for details concerning a hypoglycemic infant. The abbreviations are: C2, acetylcarnitine; C16, C16-carnitine; C18:1, C18:1-carnitine.

and individuals who are not affected but have elevations of the diagnostic values well above normal. Multiple specimens and other means of differential diagnosis are important in confirming a suspected diagnosis in atypical cases.

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