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Isovalerylglycine, isovalerylcarnitine, pivaloylcarnitine, isovaleric acidemia, antibiotics

Abstract

Background: Recent neonatal screening for isovaleric acidemia by tandem mass spectrometry based on dried blood spot levels of C5-acylcarnitines, including isovalerylcarnitine and its isomer, pivaloylcarnitine, which is derived from pivalate-generating antibiotics, has caused many false-positive results. We have developed a method to overcome this interference.

Methods: The amounts of isovalerylglycine were determined by a stable-isotope dilution electrospray tandem mass spectrometric analysis, using multiple reaction monitoring with product ions of m/z 132, which were generated predominantly from quasi-molecular ions of isovalerylglycine butylester but apparently not from those of pivaloylglycine butylester.

Results: Isovalerylglycine concentrations in dried blood spots of control newborns were 0.17 ± 0.03 nmol/ml, and those of patients with isovaleric acidemia ranged from 1.3 to 80.0 nmol/ml. Those of the newborns treated with antibiotics, which caused high C5-acylcarnitine levels (1.9 ± 1.7 nmol/ml) in dried blood spots, were 0.22 ± 0.05 nmol/ml.

Conclusions: Our data showed that the present method is useful in eliminating the false-positive results due to antibiotics use in newborn screening for isovaleric acidemia.

1. Introduction

Isovaleric acidemia (IVA; McKusick 243500) is an autosomal recessive disorder caused by mutations in the isovaleryl-CoA dehydrogenase (EC 1.3.99.10) gene. The deficiency of this enzyme in the metabolism of leucine leads to the accumulation of a series of isovaleryl-CoA metabolites, such as 3-hydroxyisovaleric acid, isovalerylglycine (IVG) [1,2], and isovalerylcarnitine [3], in body fluids. There are three phenotypes, two classic forms of the severe neonatal-onset type and the milder chronic intermittent type, and a mild biochemical form, which has been identified in newborn screening by tandem mass spectrometry (MS-MS) [4]. In newborn screening for organic acidemias and fatty acid oxidation disorders, acylcarnitines in dried blood spots (DBS) are measured by flow-injection electrospray ionization (ESI) MS-MS [5-7]. In this measurement, the isomers of isovalerylcarnitine as an indicator of IVA and pivaloylcarnitine, which is derived from pivalate-generating antibiotics (PGA), are not determined separately. Thus, the common usage of this kind of antibiotics with newborns resulted in a relatively high false-positive rate (0.5-1%) for IVA in Japan [8]. It is important to reduce false-positive results, which may place families at risk for increased stress [9]. To overcome this interference, we have developed a method to measure IVG concentrations in DBS by ESI-MS-MS for newborn screening.

2. Experiment

2.1. Materials

2.1.1. Biological samples

The dried blood spots used in our pilot study of newborn screening by MS-MS were collected on the newborns' fifth or sixth day of life, with the informed consent of their parents, using the same filter paper (Toyo Roshi, Tokyo, Japan) as that used in general neonatal screening in Japan [6].

A Japanese patient with IVA was diagnosed prenatally because of his elder siblings with this disease [10]. The other three patients with IVA became symptomatic in their early infancy and were diagnosed by urinary organic acid analysis using gas chromatography-mass spectrometry (GC-MS) and enzyme assay [11]. Frozen serum specimens and dried

blood spots were transferred to our laboratory and stored in a freezer until analysis.

2.1.2. Chemicals

[$^2H_2$]glycine was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Isovaleryl chloride, pivaloyl chloride, 2-methylbutyric acid, thionylchloride, glycine, and HPLC-grade formic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetyl chloride and n-butanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol, and distilled water were purchased from Nacalai Tesque (Kyoto, Japan). IVG, [$^2H_2$]IVG, pivaloylglycine (PG), [$^2H_2$]PG, and 2-methylbutyrylglycine (MBG) were synthesized according to the reported methods [12].

2.2. Methods

2.2.1. Sample preparation

To the dried blood-spot punch (3.2 mm diameter) in a well of a microtiter plate, 110 μl of 99% methanol solution containing 0.2 nmol/ml of [$^2H_2$]IVG was added, and the plate was agitated gently at room temperature for 15 min. The extract solution was transferred to a second plate and dried under a nitrogen stream. The dried residue was derivatized at 65˚C for 15 min using 3N HCl/butanol. The derivatized sample was dried under a nitrogen stream and dissolved in 80% acetonitrile solution with 0.05% formic acid.

For calibrators, aliquots of heparinized whole blood of a healthy adult were fortified with IVG at blood concentrations of 0.10, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50 nmol/ml, and each of the aliquots was spotted on the filter paper mentioned above.

To determine the recovery rate of IVG, the calibrator with 5 nmol/ml of IVG was extracted using 110 μl of methanol solution without [$^2H_2$]IVG, and the extract was transferred into a well containing 110 μl of methanol solution with [$^2H_2$]IVG and then processed using the method mentioned above. The values obtained were compared with those of the solution containing IVG and [$^2H_2$]IVG processed without extraction.

To determine the IVG concentrations in serum samples, the mixture of 5 μl of serum and 183 μl of 95% methanol solution containing 0.2 nmol/ml of [$^2H_2$]IVG was centrifuged at 10,000 x g for 10 min. The supernatant was dried under a nitrogen stream, and the dry

residue was derivatized as mentioned above.

The acylcarnitines and free carnitine in sera and DBS were measured according to the methods reported previously [6,8].

2.2.2. Tandem mass spectrometry conditions

A model API 4000 triple-stage mass spectrometer (Applied Biosystems, Tokyo, Japan) was used as an MS-MS system, equipped with a Turbo Ion Spray® as ESI. The heated gas temperature was 500˚C, and the ion source voltage was 5500eV. Using a model LC10Avp HPLC system and a model SIL-20AC auto-injector (Shimadzu, Kyoto, Japan), derivatized samples (13 μl) were injected at about 0.8-min intervals into a flow of the mixture of 95% methanol and 90% acetonitrile (1:1, v/v) with 0.05% formic acid. The flow rate was programmed as follows: 1.0 ml/min from 0 to 0.03 min, 0.05 ml/min from 0.04 to 0.4 min, and 1.5 ml/min from 0.41 min. The mass spectrometer was operated using Analyst software version 1.4. The quadrupole operated at unit resolution. Suitable measurement conditions for designated transitions from precursor to product ions were identified with the automatic tune function of the Analyst software. The positive ion MS-MS was performed in multiple-reaction monitoring (MRM) mode with m/z 216 $\rightarrow$ 132 for IVG, m/z 216 $\rightarrow$ 142 for IVG and PG, and m/z 218 $\rightarrow$ 134 for [$^2H_2$]IVG with declustering potential of 51 V, collision energy of 15 V, and collision cell exit potential of 8 V. Collision gas (nitrogen) parameter was the original value of the instrument. The data were recorded for 0.4 min after every sample injection. For quantification, the recorded intensities of the designated MRM ion set, which were higher than 30% of the highest, were averaged using the Chemoview software.

3. Results

Figure 1 shows the product ion mass spectra of quasi-molecular ions (m/z 216) of derivatized IVG, MBG, and PG under the MS-MS conditions suitable for derivatized IVG mentioned above. The product ion m/z 132 was the highest, and that of m/z 142 was the second highest in intensity among those of quasi-molecular ions of derivatized IVG and MBG, while that of m/z 142 was the highest, and that of m/z 132 was markedly low among

those of quasi-molecular ions of derivatized PG. MRM chromatograms in the analysis of a patient with IVA and a newborn treated with PGA are shown in Fig. 2. The increased intensity of m/z 216 → 132 in the measurement of the patient sample demonstrated the accumulated isovalerylglycine. The increased intensity of m/z 216 → 142 indicated the accumulated PG in a newborn treated with PGA. Quantification was performed using averaged intensities of the designated MRM ion set.

The averaged intensity of MRM measurements with m/z 216 → 132 of methanol solution containing 0.2 nmol/ml of [$^2H_2$]IVG without extraction was 2.1 (± 0.2) x 10^2 cps, and those of DBS containing 0 and 0.1 nmol/ml of spiked IVG, extracted with methanol solution containing 0.2 nmol/ml of [$^2H_2$]IVG, were 3.2 (± 0.5) x 10^2 and 5.1 (± 0.4) x 10^2 cps, respectively. The linearity of the calibration curve for spiked IVG in DBS was observed over the concentration range 0.10-50 nmol/ml: slope = 1.12, intercept = -0.14 in X-axis of the IVG value obtained using the intensity ratio. The mean recovery for IVG was 87% at the concentration of 5 nmol/ml in calibrators.

The intra-assay variabilities were tested by the analysis of six samples processed at the same time, and the inter-assay variabilities were tested by processing and analyzing one sample on 6 different days, using the DBS calibrators. The coefficients of variation are listed in Table 1.

IVG concentrations in DBS of the patients with isovaleric acidemia, the newborns treated with PGA, and control newborns are shown in Table 2. The value obtained by MRM with m/z 216 → 132 using DBS spiked with PG at the concentration of 50 nmol/ml was 0.80 ± 0.05 nmol/ml (mean ± S.D.; n=6).

The serum levels of IVG and isovalerylcarnitine of a patient with IVA diagnosed prenatally are shown in Fig. 3. The concentration of IVG was markedly high (8.7 nmol/ml) in his first day of life and higher than that of isovalerylcarnitine. It reached the highest value two days after birth then decreased. The levels of isovalerylcarnitine exceeded those of IVG after carnitine therapy.

4. Discussion

Diagnosis of IVA was classically based on the detection of massive excretion of a

series of isovaleryl-CoA metabolites, including IVG, in the urine of symptomatic patients, by the use of GC-MS. However, recent application of MS-MS to newborn screening has brought a new era when the majority of patients with IVA are diagnosed pre-symptomatically through newborn screening in the United States and several European countries [13]. In MS-MS newborn screening for IVA, the marker metabolite is C5-acylcarnitine in DBS, which represents a mixture of isomers (isovalerylcarnitine, 2-methylbutyrylcarnitine, and pivaloylcarnitine), and cannot be measured separately by flow-injection ESI-MS-MS. Thus, the newborns with elevated C5-acylcarnitine in DBS have to be evaluated further by GC-MS analysis of urine specimens even though elevated C5-acylcarnitine in symptomatic infants, determined by MS-MS, is a diagnostic indication of IVA [14].

Pivaloylcarnitine excretion in humans after PGA treatment was reported using GC-MS and fast-atom bombardment MS in 1987 [15]. A false-positive result for IVA in newborn screening by MS-MS was first reported in Argentina in 1998; the newborn was breast-fed by a mother treated with PGA [16]. We have been disturbed by a lot of false-positive results due to PVA administration to newborns since the start of our pilot study of MS-MS newborn screening in 1997 [6].

In the present study, we have shown that the concentrations of IVG in DBS and serum specimens can be determined accurately by flow-injection ESI-MS-MS, where the interference by PG was negligible. The proposed structural formulae of product ions of m/z 132 and m/z 142 are shown in Fig. 4. It is thought that the low intensity of product ion of m/z 132 derived from quasi-molecular ion of derivatized PG may be due to the preferential formation of product ion of m/z 198 through the loss of H_2O.

Although the sensitivity was not high enough to determine the exact IVG levels in control newborns based on the noise intensities, our tentative IVG cut-off value of 0.5 nmol/ml for screening of IVA patients was adopted, based on the data shown in Table 2. In our IVA patients with classic forms, the DBS levels of IVG were much higher than our cut-off value and higher than those of C5-acylcarnitine. In our patient with the severe neonatal-onset type, the IVG levels in serum specimens were markedly high at 21.2 nmol/ml in his early newborn period, even under intravenous glucose infusion. On the other hand, in our

patient with a milder type of IVA, the DBS concentration of IVG was 2/3 of that of C5-acylcarnitine, and the lowest level of newborn DBS C5-acylcarnitine in IVA patients with a mild biochemical form was reported to be 0.8 nmol/ml [4]. Thus, our cut-off value should be reconfirmed in our future analysis.

We are now using our method to determine IVG in DBS in a second-tier analysis of the cases with high C5-acylcarnitine since we are measuring acylcarnitines and amino acids in DBS without derivatization in routine MS-MS newborn screening. However, the present methods can be applied to the routine screening using derivatized samples although the sensitivity of the instrument might be crucial.

We have shown the possible interference of another isomer of MBG, which is detected in the urine of patients with a very rare disorder of short/branched-chain acyl-CoA dehydrogenase deficiency [17]. It is reported that the levels of MBG in urine and C5-acylcarnitine in DBS of the patients with this disorder were as low as those of IVG and C5-acylcarnitine in the patients with a mild biochemical form of IVA. Thus, the patients with this disorder may be identified based on the increased levels of IVG determined by the present method, which indicates that urinary organic acid analysis is necessary as a confirmatory test in the case of high IVG in DBS.

In conclusion, the present method is useful to eliminate the false-positive results due to antibiotics use in MS-MS newborn screening for isovaleric acidemia.

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List of abbreviations

DBS; dried blood spots

ESI; electrospray ionization

GC-MS; gas chromatography-mass spectrometry

IVA ; Isovaleric acidemia

IVG; isovalerylglycine

MRM; multiple-reaction monitoring

MS-MS; tandem mass spectrometry

PGA; pivalate-generating antibiotics

PG; pivaloylglycine

MBG; 2-methylbutyrylglycine.

Legends

Fig. 1. Mass spectra of the product ions derived from quasi-molecular ions ($[M+H]^+$) of butyrated isovalerylglycine [A], 2-methylbutyrylglycine [B] and pivaloylglycine [C]. The intensity of product ion m/z 132 was high in [A] or [B], but very low in [C].

Fig. 2. Multiple-reaction monitoring chromatograms in the measurements of DBS samples of a patient with isovaleric acidemia [A] and a newborn treated with pivalate-generating antibiotics [B]; m/z 216 $\rightarrow$ 132 for isovalerylglycine, m/z 218 $\rightarrow$ 134 for [$^2H_2$]isovalerylglycine, and m/z 216 $\rightarrow$ 142 for pivaloylglycine and isovalerylglycine. Quantification was performed using averaged intensities of the designated MRM ion set. The increased intensity of m/z 216 $\rightarrow$ 132 in [A] demonstrated the accumulated isovalerylglycine in a patient with isovaleric acidemia.

Fig. 3. Serum levels of isovalerylglycine, isovalerylcarnitine, and free carnitine in a patient with isovaleric acidemia who was treated with intravenous infusion of glucose immediately after birth because of a prenatal diagnosis of this disease. The isovalerylglycine levels reached the highest value two days after birth then decreased. The levels of C5-acylcarnitine exceeded those of isovalerylglycine after carnitine therapy. See text in regard to clinical information of this patient.

Fig. 4. Proposed structural formulae of product ions of m/z 132 and m/z 142. In pivaloylglycine butylester, the preferential formation of ring-form product ion of m/z 198 by the loss of H_2O may cause the paucity of the formation of product ion m/z 132, as shown in Fig.1.

Fig.1

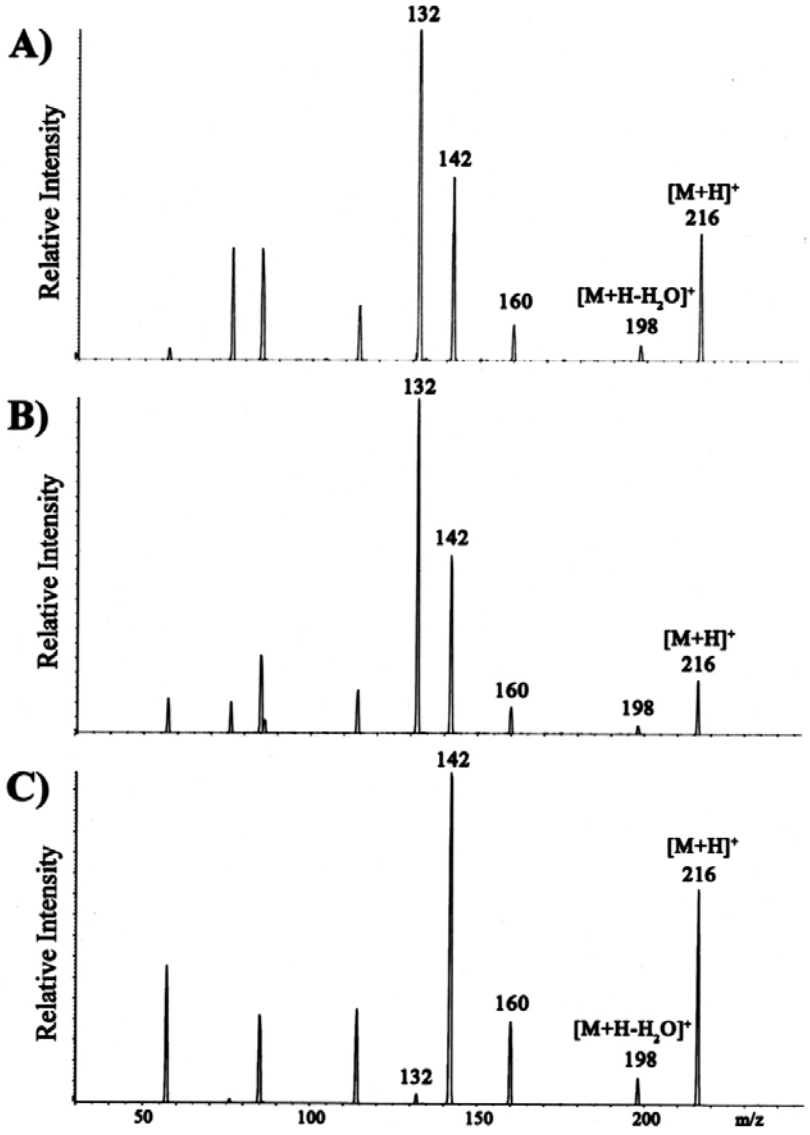
A)
Relative Intensity
132
142
160
[M+H]+
216
[M+H-H2O]+
198
B)
Relative Intensity
132
142
160
198
[M+H]+
216
C)
Relative Intensity
142
132
160
[M+H]+
216
[M+H-H2O]+
198
50
100
150
200
m/z

Fig.2

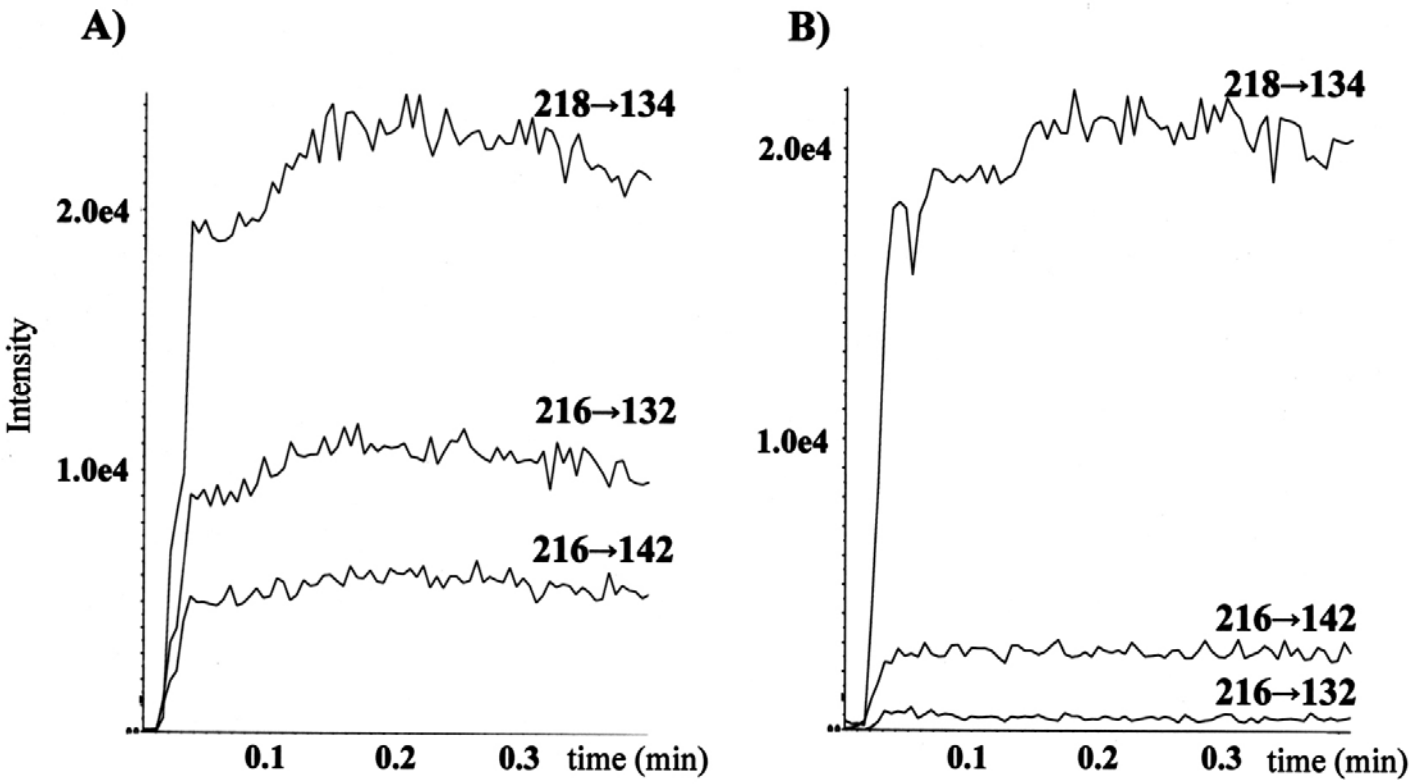
A)
218→134
2.0e4
216→132
1.0e4
216→142
Intensity
0.1
0.2
0.3
time (min)
B)
218→134
2.0e4
1.0e4
216→142
216→132
0.1
0.2
0.3
time (min)

Fig.3

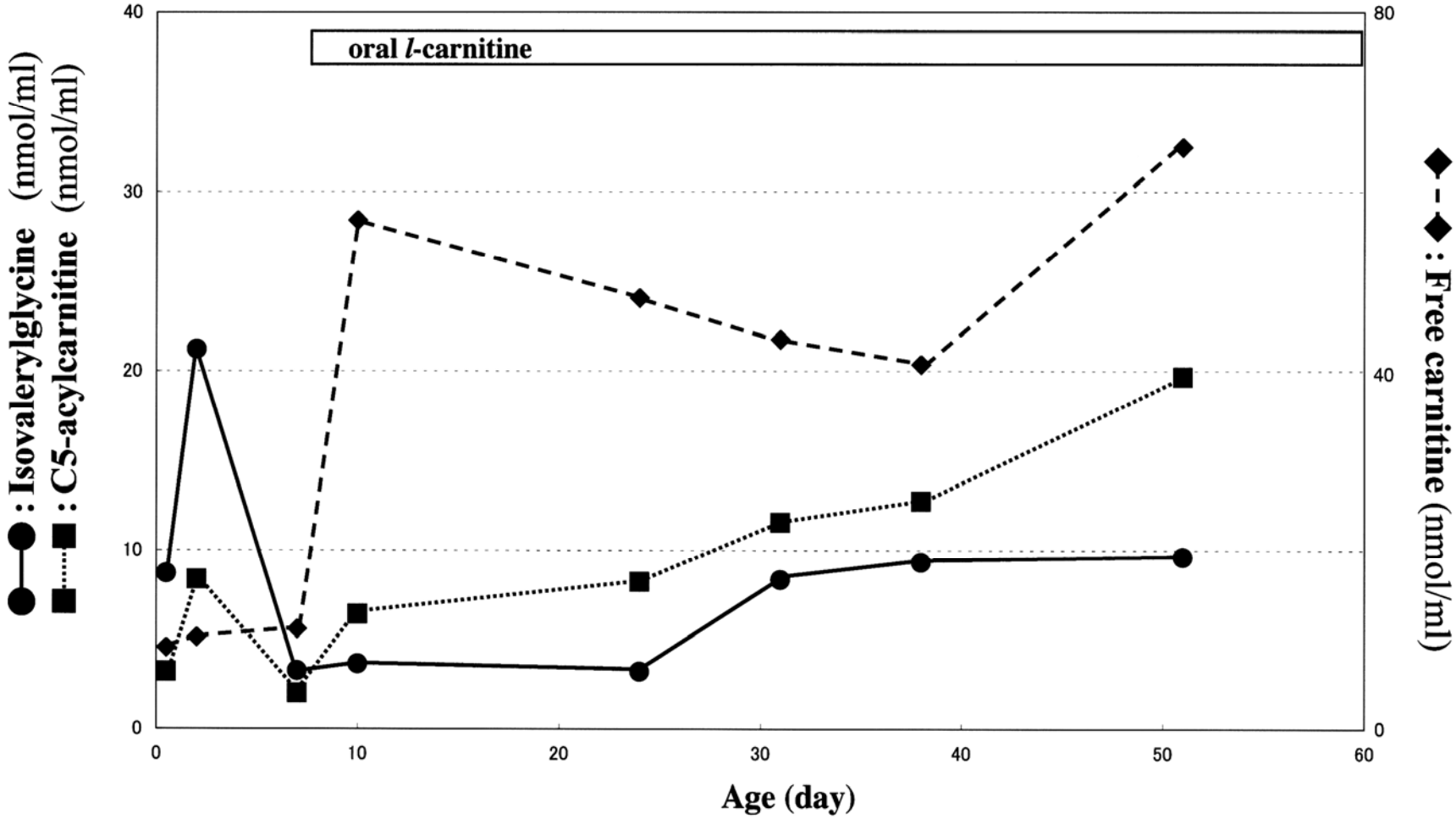
oral l-carnitine
●: Isovalerylglycine (nmol/ml)
■: C5-acylcarnitine (nmol/ml)
◆--◆: Free carnitine (nmol/ml)
Age (day)
0
10
20
30
40
50
60
80

Fig.4