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Prenatal diagnosis for organic acid disorders using two mass spectrometric methods, gas chromatography mass spectrometry and tandem mass spectrometry

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Abstract

We performed prenatal diagnosis of organic acid disorders using two mass spectrometric methods; gas chromatography mass spectrometry (GC/MS) and tandem mass spectrometry (ESI/MS/MS). Of 28 cases whose amniotic fluid was tested, 11 cases were diagnosed as "affected". All cases whose samples were diagnosed as "unaffected" were confirmed to have no symptoms or abnormalities in urinary organic acid analysis after birth. Of the 11 "affected" cases, two cases were missed by ESI/MS/MS but not by GC/MS. When the stability of metabolites in amniotic fluid was checked, it was found that acylcarnitines degraded in one week at room temperature, whereas organic acids such as methylmalonate or methylcitrate were stable for at least 14 days. Prenatal diagnosis by analysis using simultaneous two or more methods may be more reliable, though attention should be paid to sample transportation conditions.

Keywords: Prenatal diagnosis; Organic acid disorders; GC/MS; ESI/MS/MS; Stability of metabolites

1. Introduction

It is known that mass spectrometric measurements of metabolites in amniotic fluid can provide rapid and precise data in the prenatal diagnosis of organic acid disorders [1–4]. Measurements of enzyme activity in cultured amniotic cells [5] are time-consuming and troublesome. Mutation analysis using amniocytes or chorionic villi requires information on the proband, and the contamination of maternal cells may potentially lead to "false negative" results. Two kinds of mass spectrometric methods for prenatal diagnosis have been reported; one is organic acid analysis by the stable-isotope dilution method using GC/MS [3,6–9], and the other acylcarnitine analysis using ESI/MS/MS [4,10]. These methods can provide fast results with only a small amount of amniotic fluid.

We used the above two methods simultaneously, for prenatal diagnosis of 28 cases at risk of organic acid disorders. Of the 28 cases examined, 11 cases were eventually diagnosed as "affected". In two of the 11 cases however, ESI/MS/MS indicated "unaffected". Here we report the results of prenatal diagnosis using these two methods, and the stability of metabolites in the amniotic fluid determined to resolve the discrepancy which yielded the "unaffected" diagnosis results.

2. Materials and methods

2.1. Reagents

D3-methylcitrate (MC), d3-methylmalonate (MMA), d4-glutarate (GA), and d3-isovarerylglycine (IVG) were purchased from Sigma (St. Louis, MO, USA). d2-methylcrotonylglycine (MCG) was kindly provided by Prof. A. Shimizu of Osaka Medical University. *N*-methyl-*N*-(*t*-

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Table 1
Prenatal diagnosis of organic acid disorders by two methods, GC/MS and ESI/MS/MS

Disease	No of cases	Weeks of gestation (weeks)	Cases judged "affected"	
			GC/MS	ESI/MS/MS
Methylmalonic acidemia (MMA-emia)	17	12–17	7	6
Propionic acidemia (PPA-emia)	7	15–16	3	2
Multiple carboxylase deficiency	3	16–17	0	0
Glutaric acidemia type I (GA1)	1	17	1	1
Total	28		11	9

butyldimethylsilyl)trifluoroacetamide (TBDMS) was provided by Aldrich (Milwaukee, WI, USA); Dimethylformamide (DMF) from Applied Biosystems (Foster, CA, USA). All the other reagents were from Nacalai Tesque (Kyoto, Japan). Acetyl-[methyl-$^2H_3$]carnitine (d3-C2, *m/z* 263), propionyl-[methyl-$^2H_3$]carnitine (d3-C3, *m/z* 277), butyryl-[methyl-$^2H_3$]carnitine (d3-C4, *m/z* 291), and glutaryl-[methyl-$^2H_3$]$_3$carnitine (d9-C5DC, *m/z* 397) were synthesized, according to previously reported methods [11,12].

2.2. Amniotic fluid samples

We analyzed 28 cases of amniotic fluid samples at risk for organic acid disorders as listed in Table 1; 17 cases at risk for methylmalonic acidemia (MMA-emia), seven for propionic acidemia (PPA-emia), three for multiple carboxylase deficiency (MCD), and one for glutaric acidemia type I (GA1). Amniotic fluid samples were collected at 12–17 weeks of gestation. They were 20 Japanese cases and eight cases from China or India. Amniotic fluid samples from Japan were transported on dry ice, while samples from the other Asian countries were transported at room temperature, taking 4–7 days. The samples were stored at −20 °C until analysis.

2.3. Sample preparation for GC/MS

One millilitre of amniotic fluid was added with the following stable isotope-labeled compounds, 10 nmol/ml each of d3-MMA, d3-MC, d4-GA, d3-IVG and/or d2-MCG, as internal standards. The mixture was then added with 10 units of urease and incubated for 30 min. The solution was acidified with 350 μl of HCl (6 N), and the organic acid fraction was extracted twice with 10 ml each of ethylacetate. The supernatant was evaporated to dryness under a nitrogen stream at 60 °C. The dry residue was re-dissolved in a solution of 50 μl of TBDMS and 50 μl of DMF, and then the sample was incubated at 80 °C for 1 h for *tert*-butyldimethylsilyl derivertization. The concentration of the organic acids was calculated using the relative peak area of each Q-ion to that of the corresponding internal standards.

2.4. Organic acids analysis by GC/MS

A capillary GC/MS system, Shimadzu GCMS-QP 5050 Model (Shimadzu, Kyoto, Japan) equipped with a class 5000 data processing system, and capillary fused-silica DB-5 column (30 m × 0.5 mm i.d.) with a 1.0 μm film thickness of 5% phenylmethyl silicone (J & W Scientific, Folsom, CA, USA) were used. The injector temperature was maintained at 280 °C. Helium was used as the carrier gas at 100 kPa. The temperature was programmed at a rate of 4 °C/minute rising from 100 to 300 °C as reported previously. Quantification was carried out in the SIM mode. The selective ions of each organic acid for quantification and confirmation (Q-ion and C-ion, respectively) are listed in Table 2.

2.5. Sample preparation for ESI/MS/MS

The sample preparation for ESI/MS/MS analysis was done according to the method of Shigematasu et al. [4] with minor modifications. 200 μl of amniotic fluid was added with the internal standards as reported previously [4] and 1.0 ml of cold ethanol. The mixture was then centrifuged at 10,000 × *g* for 10 min. The supernatant was dried under a nitrogen stream, and the dry residue was derivatized with 100 μl of HCl (3 N) in *n*-butanol at 65 °C for 15 min. The sample was dried again under a nitrogen stream, re-dissolved in 50 μl of 50% acetonitrile, and subjected to ESI/MS/MS.

2.6. Acylcarnitines analysis by ESI/MS/MS

A model TSQ-7000 tandem quadrupole mass spectrometer (Finnigan MAT Instrument Inc., Tokyo, Japan) was used. Aliquots of each sample were injected manually with a 7725 injector (Rheodyne, Cotati, CA, USA), and introduced into the ESI interface through a deactivated fused silica capillary

Table 2
Selective ions of index metabolites used in stable isotope dilution analysis using GC/MS

Compound	Q-ion (*m/z*)	C-ion (*m/z*)
d0-Methylmalonate	289	189
d3-Methylmalonate	292	189
d0-Methylcitrate	605	473
d3-Methylcitrate	608	476
d0-Gltutarate	303	189
d4-Gltutarate	307	189
d0-Methylcrotonylglycine	214	83
d2-Methylcrotonylglycine	216	83
d0-Isovalerylglycine	216	132
d3-Isovalerylglycine	219	132

Abbreviations: Q-and C-ion = selected ion for quantification and confirmation, respectively.

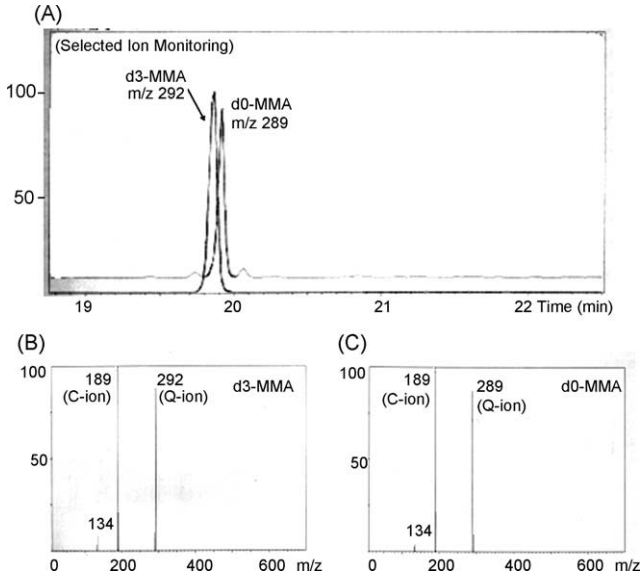

Fig. 1. Mass chromatograms and mass spectrum of *t*-BDMS derivatives of methylmalonate (MMA) and stable-isotope-labeled MMA in case at risk for methylmalonic acidemia. (A) Mass chromatogram of MMA (*m*/*z* 289) and d3-MMA (*m*/*z* 292). (B) and (C) Mass spectrum of MMA and d3-MMA, respectively.

column (0.10 mm i.d., 30 cm length) using a 50% acetonitrile flow (25 μl/min) of a model LC-10AD liquid chromatograph (Shimadzu, Kyoto, Japan).

The analytical conditions of ESI/MS/MS were as described previously [4]. The ESI voltage was 4.5 kV, and the capillary temperature was kept at 200 °C. Argon was used as the collision gas in the second quadrupole at a pressure of 0.5 milliTorr and 30 eV collision energy. The scan range and speed of the first quadrupole were *m*/*z* 200–400 per 3 s. All acylcarnitines were measured by the data in the precursor-ion scanning with product ion *m*/*z* 85. Quantitative analysis of each acylcarnitine was performed using the ratio of the averaged ion intensity to that of the corresponding internal standards.

2.7. Stability of metabolites in amniotic fluid

The stability of organic acids and acylcarnitines in amniotic fluid from four cases of MMA-emia at room temperature was determined on days 0, 3, 7 and 14.

3. Results

3.1. Results of GC/MS and ESI/MS/MS

Mass spectra and Mass fragmentgram of TBDMS derivatives of MMA from the MMA-emia affected amniotic fluid are illustrated in Fig. 1. The peaks of all organic acids tested were detected without any non-specific peaks.

Of the 28 cases at risk for organic acidemia, seven cases were finally judged as affected with MMA-emia, three cases were diagnosed as PPA-emia and one was GA1 as shown in Table 3. In the cases affected with MMA-emia, the concentration of both MMA and MC was raised, while in case of PPA-emia, the elevation of MC only was observed. One case at risk for GA1 demonstrated a remarkable increased level of GA. The other 17 pregnancy cases did not show any significant elevation of organic acids, and were judged to be unaffected.

Table 3
Organic acid levels in amniotic fluid

		MMA	MC	GA
"Affected" case ($N = 11$)				
MMA-emia	(7)	10.98–67.17	3.62–15.15	–
PPA-emia	(3)	029–1.69	7.10–29 60	–
GA1	(1)	1.70	0.95	25.90
"Unaffected" case	($N = 17$)	0.53–6.92	0.28–1.03	–
Control	($N = 25$)	0.30–3.70	0.18–1.56	2.01–7.19

Unit: nmol/ml. Abbreviations: MMA = methylmalonate; MC = methylcitrate; GA = glutarate; MMA-emia = methylmalonic acidemia; PPA-emia = propionic acidemia; and GA1 = glutaric aciduria type I.

Fig. 2 shows a mass spectrum of acylcarnitines from a case affected with GA1. The ion intensity of glutarylcarnitine (C5DC) was significantly high, compared with that of an unaffected case.

Of the 28 pregnancies in the ESI/MS/MS analysis, eight cases exhibited the profiles suggesting "MMA-emia or PPA-emia affected", and one GA1 (Table 4). It was concluded that there were six cases of MMA-emia, and two cases of PPA-emia based on information of the probands. Although the ion intensity of propionylcarnitine (C3) in the amniotic fluids from fetuses affected with MMA-emia was relatively smaller compared with those affected with PPA-emia, a significant elevation of C3 was observed. However, using the ratios of C3 to butyrylcarnitine (C4) levels, the cases that were affected were more clearly distinguishable from the unaffected. There were two cases for which GC/MS analysis suggested affected with MMA-emia and PPA-emia respectively, whereas ESI/MS/MS indicated unaffected (Fig. 3).

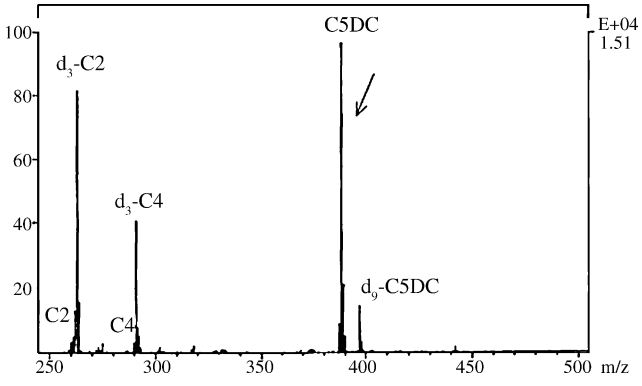

Fig. 2. Mass spectra of acylcarnitines in affected case for Glutaric acidemia type I. The peaks are the mass values of the molecular ions of the acylcarnitine butyl esters. C2 = acetylcarnitine (*m*/*z* 260); C4 = butyrylcarnitine (*m*/*z* 288); and C5DC = glutarylcarnitine (*m*/*z* 388). d_3-C2 (*m*/*z* 263), d_3-C4 (*m*/*z* 291), and d_9-C5DC (*m*/*z* 397) are deuterium-labeled used as internal standards.

Table 4
Acylcarnitine levels in amniotic fluid

		C3	C3/C4	C5DC
"Affected" case ($N = 9$)				
MMA-emia	(6)	3.90–11.20	8.90–15.50	–
PPA-emia	(2)	12.20, 16.50	1650, 21.60	–
GA1	(1)	–	–	2.98
"Unaffected" case	($N = 19$)	0.49–4.20	0.67–3.20	–
Control		0.43–1.72	1.72–2.23	0.02–0.08

Unit: nmol/ml for C3 and C5DC. Abbreviations: C3 = propionylcarnitine; C3/C4 = propionylcarnitine/butyrylcarnitine ratio; C5DC = glutarylcarnitine; MMA-emia = methylmalonic acidemia; PPA-emia = propionic acidemia; and GA1 = glutaric aciduria type I.

Twelve babies of the 17 pregnancies judged as "unaffected" could be followed and were all alive and healthy after birth. In ten, urine samples were available, and it was confirmed that the levels of organic acids were in normal range by GC/MS analysis.

Discrepancy in the results was seen in two cases between GC/MS and ESI/MS/MS. One case was judged as affected with MMA-emia and one with PPA-emia by GC/MS, whereas they were indicated as unaffected by ESI/MS/MS (Table 1, Fig. 3). In both cases, MMA-emia and PPA-emia were from China and India respectively, and the samples were sent at room temperature. In consideration of the instability of acylcarnitine, we diagnosed the two cases as "affected". In one case diagnosed as MMA-emia, we were able to measure the activity of methylmalonyl-CoA mutase using fetal skin fibroblasts, and the activity was less than 1% of control value.

3.2. Stability of metabolites in amniotic fluid at room temperature

The samples were sent from China and India at room temperature by airmail, and the turnaround time was approximately seven days. The stability of metabolites in the amniotic fluid at room temperature was determined using

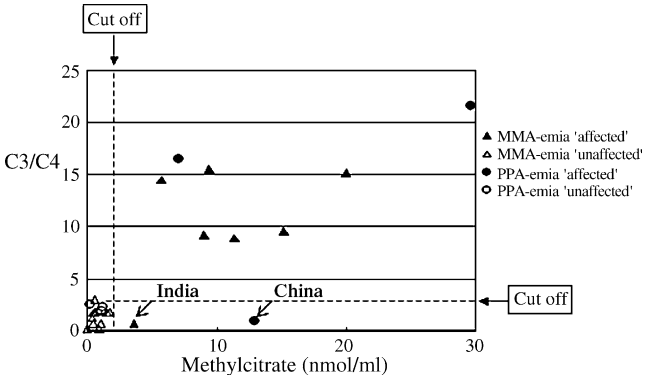

Fig. 3. Relationship between levels of methylcitrate and ratio of C3/C4 acylcarnitines (propionylcarnitine/butyrylcarnitine) in amniotic fluid for prenatal diagnosis in pregnancies at risk for propionic acidemia (PPA-emia) and methylmalonic acidemia (MMA-emia). India and China = amniotic fluid samples from India and China, respectively.

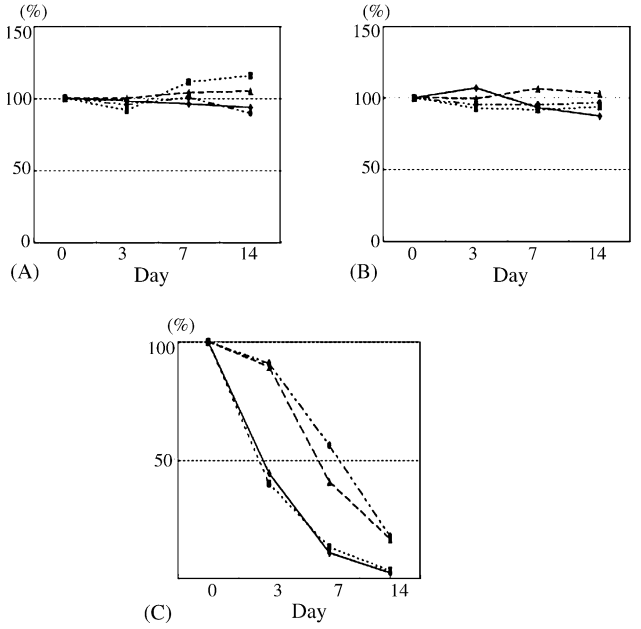

Fig. 4. Stability of methylmalonate, methylcitrate and propionylcarnitine in amniotic fluid at room temperature. Samples used were from four cases affected with methylmalonic acidemia. Percentage change of methylmalonate (A), methylcitrate (B) and propionylcarnitine (C) on days 0, 3, 7 and 14 is plotted.

amniotic fluid samples from four cases of MMA-emia that were analyzed on days 0, 3, 7 and 14. In GC/MS analysis, the relative peak area values (RPA, %) of MMA and MC on the mass fragmentogram at the starting point (day 0) was converted to "100", and compared for their percentage changes. In ESI/MS/MS, acetylcarnitine (C2), C3 and C4, and the C3/C4 ratio were determined. The ratio of the ion intensity of the acylcarnitines to that of the corresponding internal standards on the mass spectra at day 0 was converted to "100" and evaluated for stability.

As shown in Fig. 4, MMA and MC were found to be relatively stable, with values over 85% of the starting point up to at least day 14 in GC/MS analysis. By contrast, acylcarnitine was probably unstable. The C3 levels of all four cases decreased to less than about 50% on day 7, and to less than 25% on day 14, and the C3/C4 ratio was not adequate to discriminate affected cases from unaffected in two samples (data not shown).

4. Discussion

Prenatal diagnosis may include ethical considerations, and we have to be especially careful in making the final diagnosis. It may be desirable to make the final diagnosis based on several different measurements. Approaches to prenatal diagnosis include measurement of metabolites in the supernatant of the amniotic fluid as adopted in this study [3,4,13], and measurements of enzyme activity or gene mutation analysis using chorionic villi or cultured amniocytes [5,14]. The mea-

surement of the metabolites in the supernatant is fast, useful and practical.

We used two methods simultaneously, GC/MS and ESI/MS/MS, to measure metabolites in the supernatant of the amniotic fluid for prenatal diagnosis. We measured the levels of MC, MMA, GA and MCG in the amniotic fluid by the stable isotope dilution method in the SIM mode of GC/MS. Using ESI/MS/MS, C2-, C3-, C4- and C5DC-acylcarnitine levels in the amniotic fluid were measured. In general, differential diagnosis of MMA-emia and PPA-emia is difficult with the ESI/MS/MS method alone. However, there are no significant practical problems in many cases, because disease of the probands is clear.

In cases with MMA-emia or PPA-emia however, the level of C3 was sometimes in an ambiguous range. In this study, as reported previously [4], we calculated the C3/C4 ratio as well as measured the level of C3 to minimize overlapping of, and enable clarification between affected and unaffected cases.

In ESI/MS/MS, two affected cases were missed in this study. The discrepancy in these two cases turned out to be due to the instability of acylcarnitines. In fact, it was found that acylcarnitines in the amniotic fluid are rather unstable at room temperature, while organic acids are likely to be stable for at least 14 days. There have been no reports indicating the instability of acylcarnitines in the amniotic fluid.

The usefulness of blood filter paper is widely accepted in organic acidemia screening by ESI/MS/MS. We soaked amniotic fluid samples into filter paper and analyzed them. However, acylcarnitines in the amniotic fluid on the filter paper were also unstable at room temperature (data not shown). To obtain reliable results of acylcarnitines, it is necessary to transport the amniotic fluid samples on dry ice.

In conclusion, prenatal diagnosis of organic acidemias using GC/MS and ESI/MS/MS simultaneously is more useful and reliable, and the transportation of amniotic fluids for prenatal diagnosis should be handled with care.

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