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Rapid and sensitive method for prenatal diagnosis of propionic acidemia using stable isotope dilution gas chromatography–mass spectrometry and urease pretreatment

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Abstract

Propionic acidemia is one of the most frequent inborn errors of metabolism caused by a deficiency of propionyl-CoA carboxylase. Methylcitric acid, a key indicator of this disorder, is increased in amniotic fluid when a fetus is affected. Therefore, the direct chemical analysis of cell-free amniotic fluid for methylcitric acid, using stable isotope dilution gas chromatography–mass spectrometry, was carried out for the prenatal diagnosis of propionic acidemia. We developed a simple, highly sensitive, and accurate method for quantitation of this polar methylcitric acid in amniotic fluids by applying a simplified urease pretreatment which we devised earlier for urine. As the recovery of methylcitric acid from amniotic fluid was as high as 91% with a coefficient of variation lower than 3% in this procedure, only 0.02 ml of sample was required for the analysis of the affected fetus. This new procedure takes 1 h for sample pretreatment, including derivatization, and 15 min for GC–MS measurement and provides final results within 1.5 h.

Keywords: Propionic acidemia; Prenatal diagnosis; Urease pretreatment; Methylcitric acid

1. Introduction

Propionic acidemia (PCCD), one of the most frequent inborn errors of metabolism (IEM), is caused by a deficiency of propionyl-CoA carboxylase (PCC, EC 6.4.1.3). This disease leads to severe illness, physical or mental handicap and even death in early life [1]. The accurate diagnosis of PCCD is carried out by gas chromatography–mass spectrometry (GC–MS). Once the index case has been identified in a family, the availability of reliable methods for prenatal diagnosis of potential fetuses becomes an important component of genetic counseling for the family. The prenatal diagnosis of PCCD has been based on measurements of the incorporation of a label from [1-^{14}C] propionate into cellular protein in culture cells or direct measurements of the activity of PCC. Methods involving cell culture are time-consuming and potentially unreliable due to possible contamination from maternal cells [2]. The measurement of PCC activity in chorionic villi was also reported to give false results due to contamination by maternal tissue and discrepancies between the PCC activity and concentrations of methylcitric

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acid (MC) in amniotic fluid at the 11th and 15th pregnancy [3].

MC, an abnormal metabolite of propionyl-CoA metabolism, is elevated in amniotic fluid when the fetus is affected with PCCD. Since the direct chemical analysis of MC in cell-free amniotic fluid using the stable isotope dilution method was introduced by Naylor et al. [4] and Sweetman et al. [5], a direct method has been developed not only for PCCD [6–9] but also for the other organic acidemia [10]. This technique offers the considerable advantages of repeatable and rapid and early diagnosis.

MC is a very polar compound. A simplified urease pretreatment devised for multiple analysis of urine [11,12] is expected to provide a very high recovery of MC from amniotic fluids and shorten the preparation time. In the present paper, we report the development of a new procedure for the prenatal diagnosis of PCCD that involves a simplified urease pretreatment followed by stable isotope dilution GC–MS.

2. Materials and methods

2.1. Subjects

Cell-free amniotic fluid samples from pregnancies ''at risk'' for PCCD used in the present study had been taken by amniocentesis carried out at between 13 and 20 weeks of pregnancy and were kept at −20 °C prior to analysis. All those ''at risk'' had been diagnosed by us using conventional organic solvent extraction or the solid-phase extraction method; two affected, three unaffected. Reference samples were obtained from pregnancies followed up for possible chromosomal disorders. In each case, a familial history of metabolic disease was absent.

2.2. Reagents

MC and d_3-methylcitric acid (d_3-MC) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The purity of MC and d_3-MC was greater than 99%. Urease type C III was from Sigma (St. Louis, MO, USA). Trimethylchlorosilane (TMCS) was from Tokyo Kasei Kogyo (Tokyo, Japan). All other reagents were from Wako Pure Chemical (Tokyo, Japan).

2.3. Preparation

The method of Matsumoto and Kuhara [11,12] developed for the preparation of urine samples was followed with minor changes. Thirty or 60 units of urease solution was added to 0.02 or 0.5 ml of amniotic fluid and incubated at 37 °C for 10 min. To this reaction mixture, d_3-MC was added as an internal standard to obtain a final concentration of 10 nmol/ml. The mixture was vortexed with 0.9 or 4.5 ml of ethanol (final concentration 90%) and centrifuged for deproteinization. The supernatant was evaporated under a stream of N_2 at 37 °C. Trimethylsilylation was carried out by adding 100 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and TMCS (10:1, v/v) and heating at 80 °C for 30 min.

2.4. GC–MS

An aliquot (1 μl) of the derivatization mixture was injected into a Hewlett-Packard Model 6890/5973 gas chromatography–mass selected detector equipped with a fused-silica capillary column (J&W DB-5MS, 0.25 μm×0.25 mm×30 m) using an automatic injector with a split ratio of 10:1. All the conditions for GC–MS measurement were the same as described previously [11,12]. The selected ion monitoring method (SIM) was carried out for quantitation of MC (dwell time 60 ms).

3. Results

3.1. Quantitation

Fig. 1 shows the total ion current (TIC) chromatogram of trimethylsilyl derivatives of metabolites from an amniotic fluid specimen. In the simplified urease pretreatment, ethanol is used to remove proteins in biological samples and essentially no fractionation is carried out [11,12]. MC is a highly polar compound which has one hydroxyl and three carboxyl groups. MC is also very soluble in ethanol. Therefore, the recovery of MC is high with the

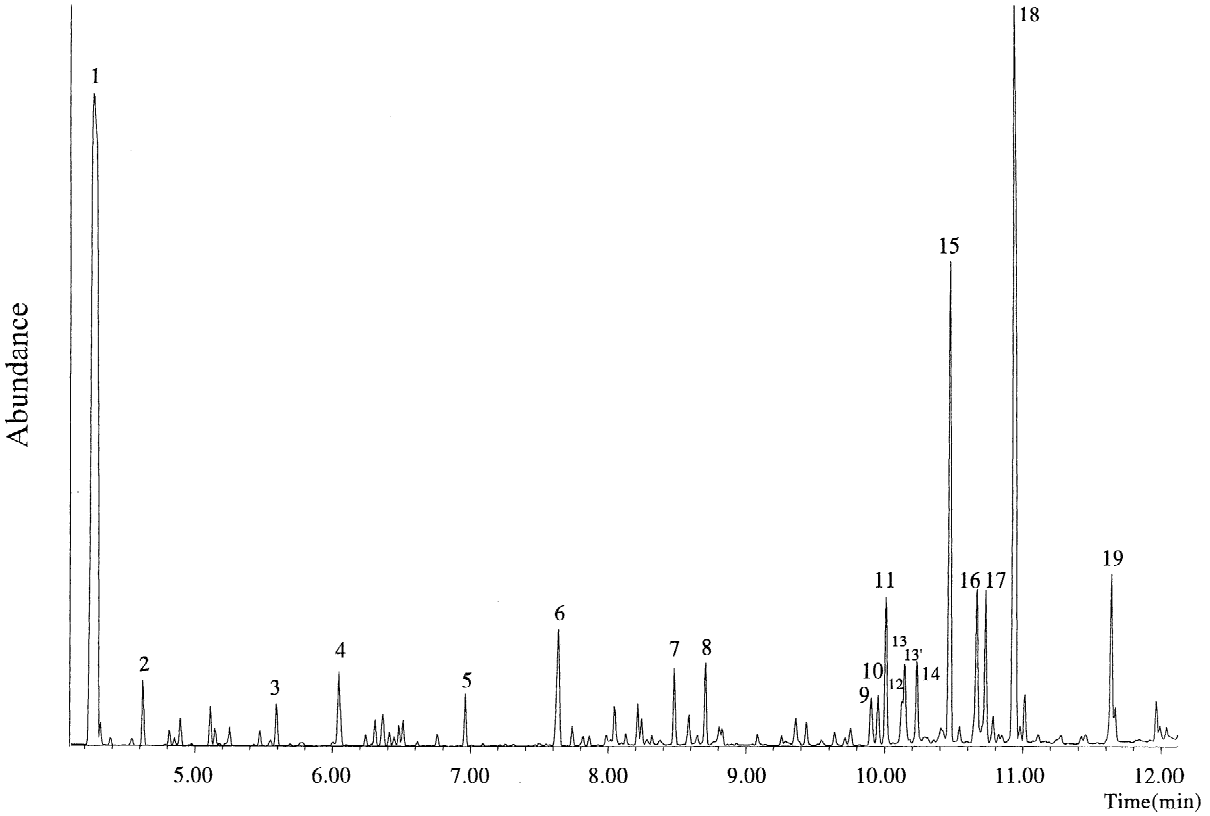

Fig. 1. TIC chromatogram of trimethylsilyl derivatives of metabolites from an amniotic fluid specimen. Peak identification: (1) lactate-2, (2) alanine-2, (3) valine-2, (4) glycerol-3, (5) threonine-3, (6) aminomalonate-3, (7) ornithine-3, (8) glutamate-3, (9) fructose-5(1), (10) fructose-5(2), (11) citrate-4, (12) methylcitrate-4(1), (13) unknown, (13′) methylcitrate-4(2), (14) galactose-5(1), (15) glucose-5(1), (16) lysine-4, (17) glucitol-6, (18) glucose-5(2), (19) *myo*-inositol-6.

accuracy and sensitivity of the quantitation being significantly improved.

The mass spectra of the tetra-trimethylsilyl derivatives (4-TMS) of MC and d_3-MC are shown in Fig. 2. In the mass spectrum of MC 4-TMS, fragment ions are observed at m/z 479 $[M-CH_3]^+$, 389 $[M-CH_3-HOTMS]^+$, 377 $[M-COOTMS]^+$, 361 $[M-CH_3-HCOOTMS]^+$, and 287 $[M-HOTMS-COOTMS]^+$. The corresponding fragment ions from d_3-MC 4-TMS are at m/z 482, 392, 380, 364 and 290, respectively. Two stereoisomers of MC are separated by GC–MS. Fig. 3 shows mass chromatograms of the fragment ions of MC 4-TMS at m/z 479, 389, and 287. Among the pairs of fragment ions from non-labeled and labeled MC, m/z 287 and 290 as well as m/z 361 and 364 showed the highest intensity. These pairs were, however, influenced by other components in the amniotic fluid (Fig. 4). The ion-pairs m/z 479 and 482, and m/z 389 and 392, although weaker than m/z 287 and 290, were free from the influence of other components. Thus, the highest mass ion-pair, m/z 479 and 482, and the next highest, m/z 389 and 392, were selected for the calculation of the concentration of MC in amniotic fluid.

The ratios of these two stereoisomer peaks of endogenous MC were examined for all the amniotic fluid samples used in the present study in order to check the correlation with the concentration, but they were shown not to correlate with the concentration of MC. The ratios were not always constant (Fig. 5). Therefore, the sum of both peaks was evaluated.

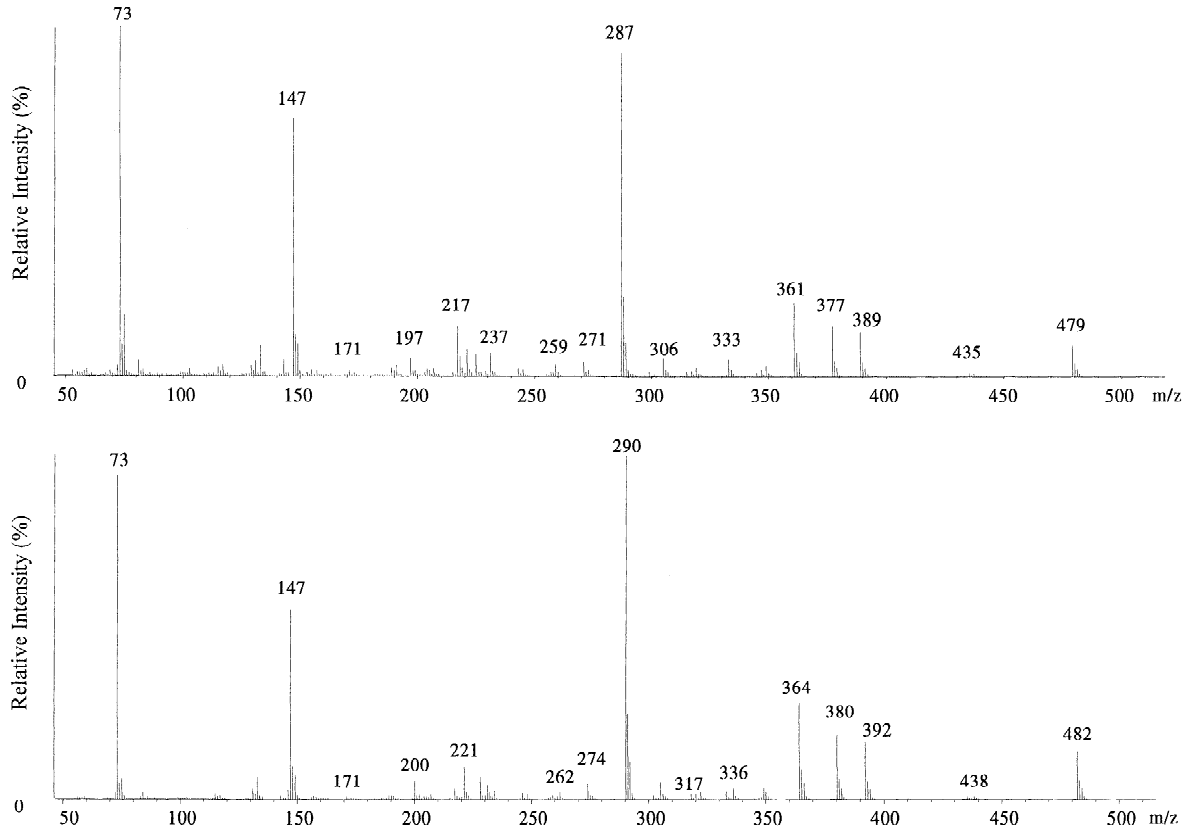

Fig. 2. Mass spectra of methylcitric acid tetra-trimethylsilyl derivatives; unlabeled (top) and methyl-d_3 labeled (bottom).

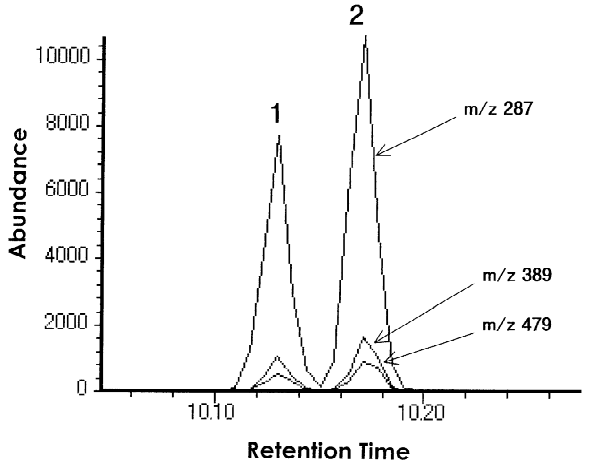

Fig. 3. Mass chromatograms for the two stereoisomers of methylcitric acid tetra-trimethylsilyl derivatives (4-TMS).

3.2. Precision and recovery

The intra-series precision of the present method was 2.5 and 3.0% coefficient of variation (C.V.) ($n=5$) at a concentration of 1.27 and 3.99 μmol/l, respectively, while the day-to-day precision was 2.2 and 2.7% C.V. ($n=4$). The inter-series precision of the method when examined using samples with 1.0 or 4.0 μmol/l MC was 3.3 and 1.1% C.V. ($n=4$). The recovery of MC was also tested with these samples. The extraction recovery of MC tested with the same samples was determined by adding MC at two known quantities, 1.0 or 4.0 nmol/ml, to a control amniotic fluid specimen and comparing the differences against the control amniotic fluid sample. The extraction recovery was 91%.

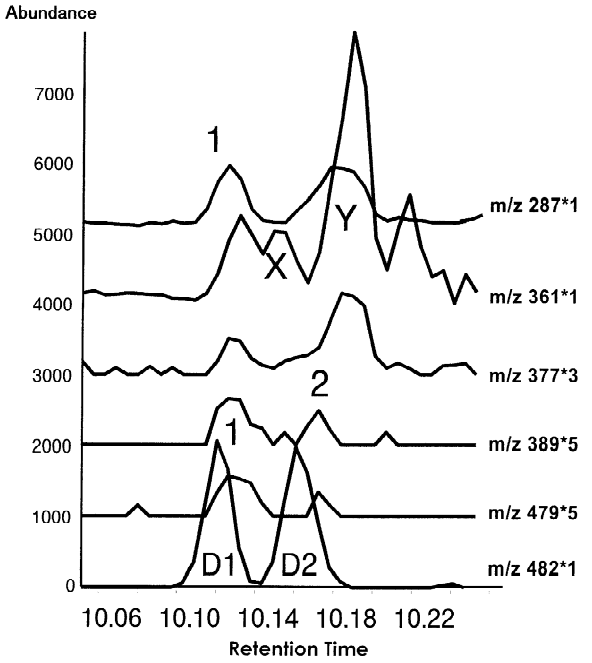

Fig. 4. Mass chromatograms for methylcitric acid 4-TMS showing that the ions at m/z 289, 361 and 377 cannot always be used for quantitation of MC in amniotic fluid specimens. 1 and 2, first and second peaks of methylcitric acid; D1 and D2, first and second peaks of d_3-methylcitric acid; X and Y, unknown peaks appearing at retention times of interest on the ion chromatogram at m/z 287, 361 and 377.

3.3. Measurement of MC in the amniotic fluid specimens of affected and unaffected fetuses

Specimens from two affected fetuses were analyzed in the present study after diluting samples 25-fold. The levels of MC in amniotic fluid from pregnancies ''at risk'' in this study are shown in Table 1, and were compared with the values measured using conventional methods. These values were similar except for the significant difference in case 1. In this case, the MC concentration was measured using heptadecanoic acid as an internal standard because d_3-MC was not obtainable at that time.

Specimens from two affected fetuses were also analyzed using scanning mode (scan range m/z 250 to 500).

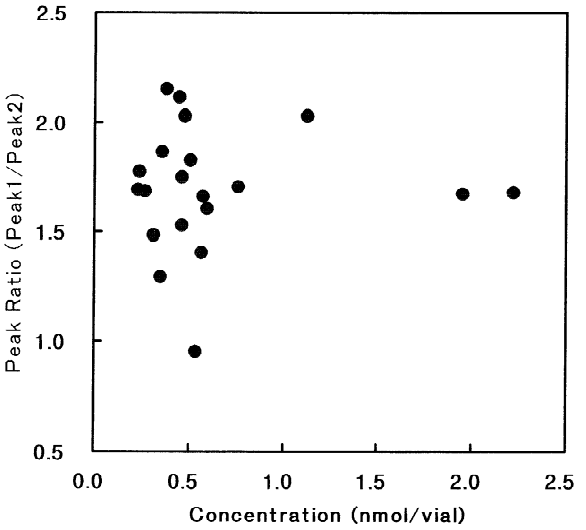

Fig. 5. Relationship between the ratio of the first and second peaks of the stereoisomers and the concentration of methylcitric acid. The average, standard deviation, and coefficient of variation of the ratio were 1.71, 0.28 and 16.4%, respectively.

4. Discussion

For the direct chemical analysis of MC in amniotic fluid, the organic solvent extraction [6] and solid-phase extraction [4,7–9] methods have been used. The simplified urease pretreatment procedure was developed for a urinary neonatal IEM screening

Table 1
Comparison of methylcitric acid concentrations in amniotic fluids ''at risk'' as measured by the present and previous procedures

Case	Concentration		''At risk''
	Present procedure	Previous procedure	
1	11.12	5.9[a]	Affected
2	8.88	7.9	Affected
3	0.57	0.4	Unaffected
4	0.60	0.6	Unaffected
5	0.46	0.4	Unaffected

Values are expressed as μmol/l.

[a] The stable isotope dilution method using d_3-methylcitric acid was not used at that time, but heptadecanoic acid was used as an internal standard.

programme [11,12]. In the present study, the urease pretreatment procedure was applied to the preparation of amniotic fluids to make a rapid and accurate prenatal diagnosis of PCCD. MC is a highly polar compound which has one hydroxyl and three carboxyl groups. It was expected that the recovery of MC from amniotic fluids into ethanol would be high. A high recovery of MC (91%) and a low C.V. (below 3%) were obtained. It was also possible to reduce the volume of the specimens to 25 μl and also that of the solvent, the number of steps, and the time required for preparation.

As shown in Table 1, it is possible to clearly distinguish between affected and unaffected fetuses by urease pretreatment, and stable isotope dilution GC–MS. In case 1 (Table 1), although we had made the correct diagnosis, the earlier measurement using organic solvent extraction was carried out without using the stable isotope dilution method and the concentration was 1.9 times lower than with the present procedure. It appears that the result had been slightly ambiguous. The use of the simplified urease procedure and the stable isotope dilution method is therefore essential for the quantitation of marker compounds in amniotic fluids for prenatal diagnosis of PCCD.

The sample volume of control amniotic fluid used in the present procedure was half of that used with the conventional method. In the three cases where the fetus was unaffected, the levels of MC were as low as the control. However, the concentrations in the amniotic fluid of affected fetuses were 20 to 30 times higher than those in the unaffected fetus. For the affected cases, 20 μl of amniotic fluid was sufficent for quantitation with SIM mode and 100 μl of that was sufficient with scanning mode.

The results presented in Table 2 show measurements of MC in amniotic fluids made in the present study together with those from the literature [7–9]. Except for a report by Fensom et al. [6] where the stable isotope dilution method was not used, the levels of MC in amniotic fluids from all affected fetuses, unaffected fetuses and controls were similar to our values. In one unaffected case in Fensom et al., in which the level of MC was higher than the mean and the activity of PCC in cultured cells was moderate, the fetus was considered heterozygous [6]. All affected fetuses had a concentration that was more than 20 times the mean for unaffected cases.

The method proposed here for the prenatal diagnosis of PCCD is rapid, highly sensitive and more accurate than methods reported previously [4–9].

Table 2
Measurement of methylcitric acid in amniotic fluid (μmol/l)

Method	Control mean±SD (*n*) (range)	At risk		Method			Gestation (weeks)	Ref.
		Affected mean±SD (*n*) (range)	Unaffected mean±SD (*n*) (range)	Sample volume (ml)	Stable isotope dilution	GC–MS column		
Urease pretreatment	0.36±0.10 (9) (0.24–0.49)	11.1, 8.88 (2)	0.54±0.05 (3) (0.46–0.60)	0.02–0.5	Y	SIM/scan capillary	13–20	Present study
Solid-phase extraction	0.30±0.10 (20) (0.15–0.50)	6.04 (4) (5.30–6.62)	0.39 (10) (0.19–0.63)	1	Y	NCI SIM capillary	12–18	[9]
Solid-phase extraction	0.38±0.10 (8) (0.24–0.58)	7.00±1.80 (17) (4.07–10.16)	0.38±0.18 (47) (0.11–1.10)	1–4	Y	NCI SIM capillary	12–20	[8]
Solid-phase extraction	0.34±0.09 (31) (0.11–0.48)	11.50, 6.80 (2)	0.43, 0.29, 0.25 (3)	1	Y	SIM capillary	14–19	[7]
Solvent extraction	<0.5	3.9, 3.6 (1)	2.4, 0.6 (1)[a]	[b]	N	Capillary	15–17	[6]

SIM, selective ion monitoring; NCI, negative chemical ionization.
[a] Amniotic fluid was obtained at 15 and 22 weeks' gestation; the fetus was diagnosed as heterozygous.
[b] No description.

This procedure does not require special solvents, columns, or special detection modes in mass spectrometry such as negative chemical ionization. The most common derivatization, trimethylsilylation, is adopted. The final results are obtained within 1.5 h.

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