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Prenatal diagnosis of propionic acidemia by measuring methylcitric acid in dried amniotic fluid on filter paper using GC/MS☆

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ABSTRACT

Propionic acidemia is a frequent inborn error of metabolism. Methylcitric acid, a key indicator of propionic acidemia, increases in the amniotic fluid of affected fetuses. For prenatal diagnosis, the methylcitric acid in amniotic fluid can be measured by stable-isotope dilution GC/MS. Here, we quantified this indicator in samples of amniotic fluid that had been dried on filter paper and transported at ambient temperatures, and compared the results with data obtained from the original amniotic fluid. We then used the filter-paper method to screen at-risk fetuses and obtained a clear-cut diagnosis in each case.

1. Introduction

It is known that an accurate and rapid prenatal diagnosis of propionic acidemia (PCCD) can be obtained using GC/MS. Until recently, the prenatal diagnosis of PCCD involved measuring the activity of propionyl-CoA carboxylase in cultured amniotic cells or chorionic villi. However, cell culture is time-consuming. Moreover, when the enzyme activity is measured in chorionic villi, the results are potentially unreliable due to possible contamination by maternal cells [1], which can lead to a false-negative conclusion [1,2].

Naylor et al. [3] and Sweetman [4] introduced the direct chemical analysis of elevated methylcitric acid (MC), an abnormal metabolite of propionyl-CoA metabolism, in cell-free amniotic fluid (AF) by GC/MS using the stable-isotope dilution GC/MS method. This method has been applied to many inborn errors of metabolism (IEMs) [5–8] and offers the considerable advantages of a repeatable, rapid, and early diagnosis.

In a previous paper [5], we established a procedure for the prenatal diagnosis of PCCD that involves a simplified urease pretreatment followed by stable-isotope dilution GC/MS. Here, we report a procedure for the prenatal diagnosis of PCCD in which AF is dried on filter paper, transported at ambient temperatures, extracted, and the MC in the samples is then measured by GC/MS.

2. Experimental

2.1. Subjects

AF samples from the amniotic sac of seven fetuses at-risk for PCCD were obtained by amniocentesis between 15 and 23 weeks of gestation and kept at −20 °C prior to analysis. The presence or absence of PCCD in all of the at-risk fetuses, three affected and four unaffected, was diagnosed by us before doing this study, using the simplified urease pretreatment, conventional organic solvent extraction, or solid-phase extraction. Control samples were obtained by amniocentesis between 15 and 16 weeks gestation from pregnancies that were followed-up for possible chromosomal disorders. There was no family history of metabolic disease in any of the control cases.

2.2. Chemicals and materials

MC and d_3-methylcitric acid (d_3-MC) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). The purity of the MC and d_3-MC was higher than 99%. Urease type C III was from Sigma Chemical Company (Saint Louis, MO). *N,O*-Bis(trimethylsilyl)-trifluoroacetamide with 10% trimethylchlorosilane (BSTFA) was from Pierce, Rockford, IL. Other reagents were from Wako Pure Chemical Industry Ltd., Osaka, Japan.

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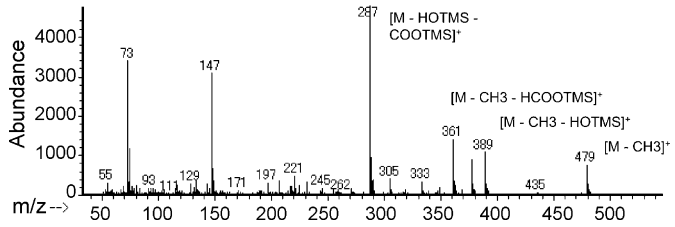

Fig. 1. Mass spectrum of authentic methylcitric acid trimethylsilyl derivatives.

2.3. Preparation

2.3.1. Average adsorption capacity of the special filter paper

The pre-weighed filter papers (B) were weighed again after they were saturated with AF (A). The unit adsorption capacity (Z) of each filter paper was calculated as follows:

$$Z = \frac{A - B}{B} \quad (1)$$

The specific gravity of AF was defined as 1.0 for this calculation. The average adsorption capacity was calculated from the unit adsorption capacity of several filter papers prepared for this study.

The volume of AF used for each experiment was calculated from the volume of extract and the average adsorption capacity of the filter paper.

The special filter paper used for dry urine samples (Advantec UA-5, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) was also used to prepare the dried AF samples. A pre-weighed filter paper was saturated in AF and then dried at room temperature. The volume of AF absorbed by the filter paper was estimated from the filter paper weight and the average adsorption capacity of this type of filter paper.

2.3.2. Preparation of sample from AF dried on filter paper

The volume of AF obtained from the dried sample was calculated by the volume of extract and the average adsorption capacity of the filter paper. The method of Matsumoto and Kuhara [9,10], developed for preparing urine samples, was followed with minor changes. The AF was extracted from the filter paper with distilled water. To 0.5 ml of the extract was added 30 units of urease solution, and the mixture was incubated at 37 °C for 10 min. d_3-MC was then added as an internal standard to a final concentration of 10 nmol/ml. The mixture was vortexed with 1.5 ml of ethanol and centrifuged for deproteinization in an Eppendorf Centrifuge 5413. The supernatant was evaporated under a stream of N_2 at 37 °C. Trimethylsilylation was performed by adding 100 μl of BSTFA and heating at 80 °C for 30 min.

2.4. GC/MS

A 1-μl aliquot 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 [5]. The selected ion monitoring method (SIM) was used for the quantification of MC (dwell times 60 ms). Fragment ions of m/z 287 [M−HOTMS-COOTMS]$^+$ and m/z 479 [M−CH_3]$^+$ were used for the quantitative and qualitative ions of MC, respectively (Fig. 1). Fragment ions of m/z 290 and m/z 482 were used for the quantitative and qualitative ions of d_3-MC. As in our previous paper [5], the summation method, in which the peak areas were added for each fragment ion pair of m/z 287 and m/z 290, was used to quantify the MC in AF (Fig. 2).

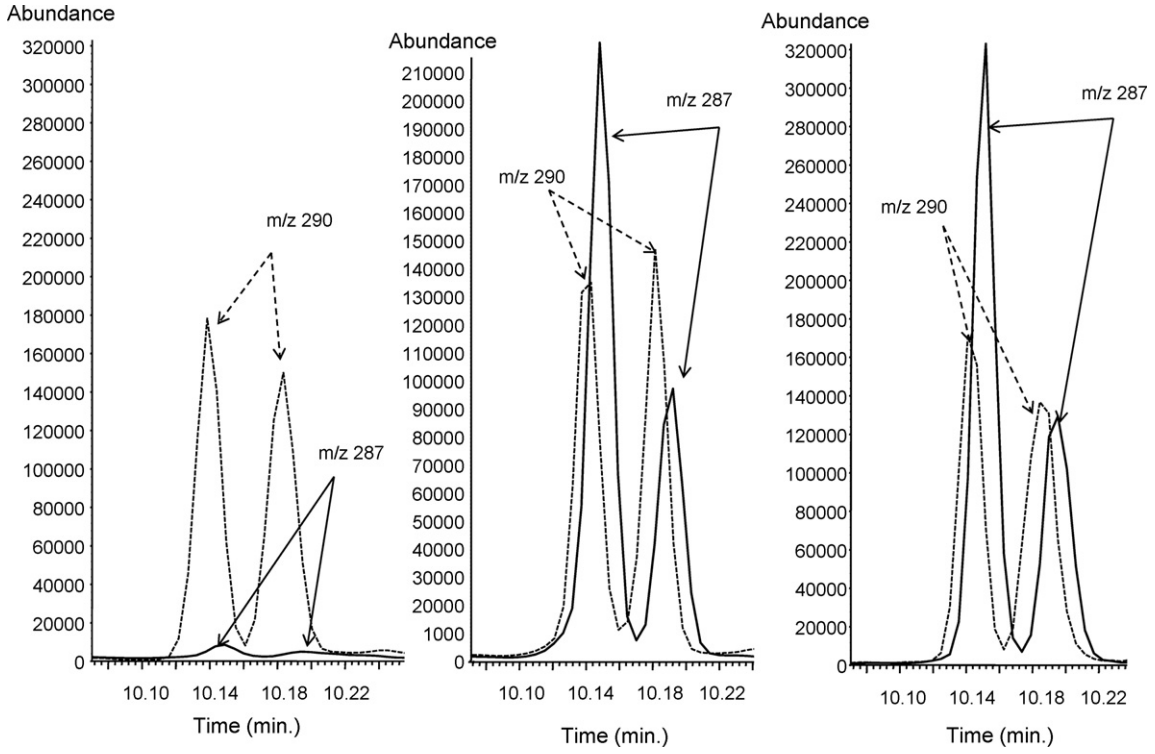

Fig. 2. Selected ion chromatograms of trimethylsilylated MC from dried filter-paper samples of control AF (left), AF from a fetus affected by PCCD (center), and the original AF sample from the same pregnancy (right). The ions of m/z 290 corresponded to the [M−HOTMS-COOTMS]$^+$ of the deuterium-labeled compound (as an internal standard).

2.5. Confirmation of AF stability on filter paper

A preparation of pooled AF samples spiked with MC to 10 nmol/ml was used to test the stability of MC in AF when stored on filter paper. Pre-weighed filter papers were saturated with this solution, then dried and stored at room temperature until the day of extraction. On the day the dried filter-paper sample was prepared (day 0), or 7 or 28 days after the preparation, the AF was extracted from the filter paper with distilled water. Each extract was prepared for GC/MS analysis as described previously [5]. The data from the samples extracted at the different time points were compared to the value of the original AF sample to determine the average recovery rate.

2.6. Reproducibility of MC measurements from AF samples dried on filter paper

To determine the reproducibility of the data obtained from the dried samples, each dried AF sample was subjected to three GC/MS measurements and averaged, and this value was compared with that of the original AF sample to obtain the recovery rate.

3. Results

3.1. Optimization of MC extraction from filter paper

In the original method designed for urine specimens, distilled water was used to extract urinary metabolites from the filter paper [9]. Because the levels of MC in AF are lower than in urine, we tried to improve the extraction efficiency. We used three different extractants to recover MC from the filter paper. The most efficient was 50% MeOH (50% aqueous solution), which had a recovery factor of 96%. The second and the third most efficient were distilled water and 30% MeOH (30% aqueous solution), with recovery factors of 91% and 86%, respectively. However, urease pretreatment is the next step, and because 50% MeOH was incompatible with the enzyme reaction conditions, it could not be used as the extractant. Therefore, distilled water was selected to elute MC from the dried AF on filter paper.

3.2. Average adsorption capacity of the filter paper

Sixteen pre-weighed filter papers were weighed again after they were saturated with AF. The unit adsorption capacity of each filter paper was calculated by Eq. (1). The average unit adsorption capacity for the 16 filter papers was 2.705. For all subsequent experiments, the presumed volume of AF was calculated from the volume of extract and this average adsorption capacity of the filter paper.

3.3. MC stability on filter paper over time

Six filter papers were saturated with a pooled AF sample spiked with 10 nmol/ml MC, and dried. On days 0, 7, and 28, three samples were prepared from each of two filter papers, and the MC was measured as described in Section 2. The average level of MC recovered from the dried filter papers at these time points was 9.8, 9.3, and 8.6 nmol/ml, respectively. The individual results from the dried AF samples, indicated as the average recovery rate of the original AF, are graphed in Fig. 3. Thus, although the level of recovered MC fell gradually over time, on day 28 it was still only slightly less than 90% of the initial value.

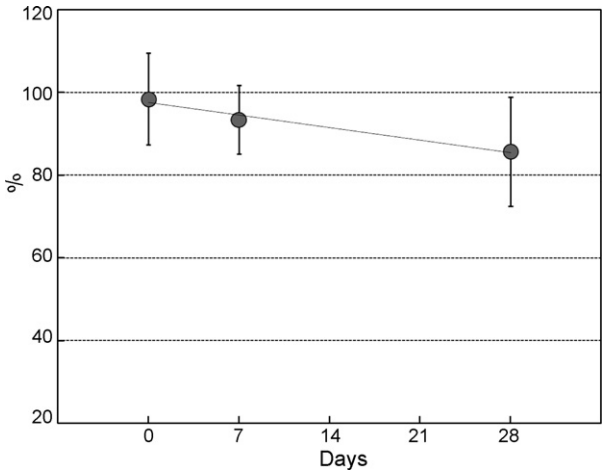

Fig. 3. Stability of methylcitric acid stored on filter paper. MC recovered on days 0, 7, or 28 after the preparation of dried AF on filter paper. Samples were stored at room temperature and eluted from the filter paper with distilled water.

3.4. Comparison of MC values in dried AF and original AF samples from control and at-risk fetuses

MC was detected in all the dried AF samples as clear peaks (Fig. 2). Three GC/MS measurements for each dried AF sample were averaged to determine the recovery rate from the original AF sample (Table 1). The average, standard deviation, maximum, and minimum recovery rates of MC from dried AF were 99.3%, 13.5%, 122.9% and 77.5%, respectively, of the original sample. The values from the dried versus original AF samples are shown graphically in Fig. 4.

Table 1 shows our findings from 10 control AF samples, and 7 samples from at-risk fetuses. The results were completely compatible with our previous diagnoses using more laborious methods. The recovery rate ranged from 78% to 123% for all measurements (Table 1). The CV% was 13.6%, and the recovery rates from the controls and unaffected cases ranged from 78% to 113% and 92% to 112%, respectively. The average and standard deviation of MC recovery rates of the 10 control dried AF samples compared with the original samples were 91.6% and 12%, respectively.

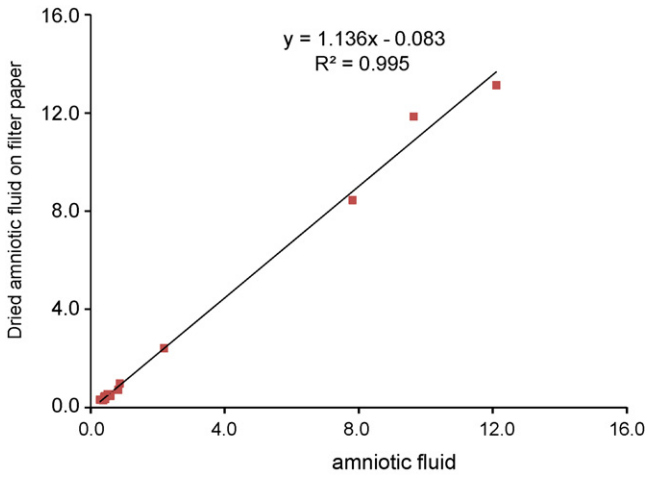

Fig. 4. Correlation between the MC levels (μmol/l) in samples extracted from dried AF on filter paper (*y*-axis) and in the corresponding original AF (*x*-axis) samples from seven at-risk and 10 control pregnancies.

Table 1
Reproducibility of methylcitric acid in amniotic fluid with a dried filter paper to that of each original amniotic fluid

Groups	No. of cases	Weeks of gestation	Original AF[a]	Dried AF[a]	Recovery rate (%)
PCCD at-risk					
Affected fetus	3	16–23	7.8–12.1	8.5–13.1	108–123
Unaffected fetus	4	15–19	0.42–0.87	0.46–0.97	92–112
Controls	10	15–16	0.28–0.82	0.29–0.72	78–113

Values are presented as minimum – maximum. PCCD, propionic acidemia (propionyl-CoA carboxylase deficiency).
[a] AF: level of methylcitric acid in amniotic fluid, μmol/l.

4. Discussion

We developed a simple, widely applicable, and highly sensitive method for quantifying MC, a polar acid, in dried AF on filter paper using the simplified urease pretreatment we reported previously [5]. To date, dry ice has been indispensable for the transportation of frozen AF samples, but the method described here circumvents that necessity, allowing samples to be sent for analysis at ambient temperatures. Even if a hospital were located in an area where dry ice is difficult to obtain, our method would permit the effective analysis of samples.

The data from the filter paper were compared with the data obtained from the original AF samples. The former is a semi-quantitative method and the latter is quantitative. The CV% of the recovery rate was 13.6%. Because this result was obtained from data calculated at different degrees of quantification, we evaluated it carefully. Our findings revealed that for measuring MC in AF, the filter-paper method was sufficiently reproducible to be applied to clinical analyses. We also analyzed the filter paper of AF from pregnancies at-risk for propionic acidemia, and were able to make a clear-cut diagnosis in each case. Therefore, even though this method is semiquantitative, it is clearly applicable to prenatal diagnosis, and is practical to carry out.

The ability to calculate the original sample volume of AF that was dried onto the filter paper was key to obtaining an accurate result using this procedure. When the calculation method presented here is used, the only considerations that must be carefully followed on-site are to make certain that the filter paper used is of known weight and that it is completely saturated with AF. The absorbed volume of the AF can then be calculated from the weight of paper.

It may seem that devising a method for calculating a fixed quantity on the basis of the change in filter-paper weight introduces an unnecessary element of uncertainty. However, it is not always easy for the on-site amniocentesis staff to spot a specific volume of AF accurately onto filter paper. Therefore, despite the drawbacks, we feel that the calculation method is a more realistic approach than one requiring the amniocentesis staff to follow a more complicated process for preparing the dried AF samples.

Since the UA-5 filter paper used for this analysis is very homogeneous, the amount of AF adsorbed per unit weight was constant. This filter paper is of moderate thickness and can absorb enough AF to analyze, and the quality of the AF, which was dried immediately after being spotted on the paper, was also sufficient for analysis.

A newborn mass-screening program using dried urine on filter paper that has continued for more than 10 years has shown that many metabolites besides MC are stable when dried on filter paper [10]. Our present stability study showed that if a dried AF sample reached an analysis center in 1 week, the result would be the same as if it had been analyzed on the day the sample was prepared. Because the MC levels are maintained at more than 80% for 4 weeks, a reliable result will be obtained even with some delay in transport or analysis. It would certainly be possible to make an accurate prenatal diagnosis if samples were sent to the analysis center by airmail. One important implication of these findings is that samples could safely be sent to an analysis center from overseas.

In the current analysis, we were able to confirm the known value of spiked MC in a pooled AF sample. We also analyzed filter papers with dried AF samples from pregnancies in which the fetus was at-risk for PCCD. The values from these measurements were reproduced dozens of times and compared with the average values from the original AF samples, which were also measured repeatedly. In every case, it was easy to make a clear-cut diagnosis. Thus, this method of sample storage, reconstitution, and analysis is accurate and practical. We hope to extend its use to the diagnosis of other IEMs from dried AF samples in the near future.

In conclusion, the method presented here is semiquantitative, but nonetheless applicable to the prenatal diagnosis of PCCD, using practical procedures on-site and at the analytical center. This method also simplifies the transportation of AF samples by eliminating the need for dry ice during shipping.

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