

# ESTABLISHING GAS CHROMATOGRAPHY - MASS SPECTROMETRY TO DIAGNOSE ORGANIC ACIDEMIAS IN THAILAND

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**Abstract.** Disorders of organic acid metabolism are a group of disorders which has long been ignored by majority of Thai physicians. Part of this is due to lack of laboratories in Thailand to verify the diagnosis of the disorders. We have recently developed a technique to qualitatively analyze organic acids utilizing Gas Chromatography - Mass Spectrometry (GC-MS). Eight patients in four families were successfully identified as having organic acidemias (OA) by this method. Two families had methylmalonic acidemia, one had propionic acidemia, and the other had 3-methylcrotonyl CoA carboxylase deficiency. To our knowledge, this is the first laboratory in Thailand being able to use GC-MS to diagnose OA. Availability of a laboratory in Thailand and affordability of the test are expected to result in earlier diagnosis and identification of more cases of OA in Southeast Asian countries. Consequently, prompt and proper treatment can be anticipated which should lead to better prognosis for patients with this group of disorder.

## INTRODUCTION

Patients presented with lethargy, hypotonia, hypertonia, tachypnea, seizures, ataxia, vomiting, failure to thrive, delayed development, and hepatomegaly may have organic acid disorders. Abnormal clinical chemistries such as cytopenia, metabolic acidosis, hyperammonemia, hypoglycemia, lactic acidemia, ketosis may also suggest abnormalities of organic acid metabolism (Goodman, 1996; Clarke, 1996). However, this group of disorders has long been ignored by many of the Thai physicians. Part of which may be due to unavailability of laboratories in Thailand to verify the diagnosis of the disorders.

The qualitative analysis of organic acids by gas chromatography - mass spectrometry (GC-MS) has well established as an important method for the diagnosis of disorders of organic acid metabolism since early 1980s (Sweetman, 1991). Here we reported accomplishment of utilizing GC-MS to identify organic acids and making diagnoses of patients with methylmalonic acidemia, propionic acidemia, and 3-methylcrotonyl CoA carboxylase deficiency. This will expedite the diagnosis of OA in Thai and other Southeast Asian patients. Therefore, prompt treatment and better prognosis can be anticipated.

## MATERIALS AND METHODS

### Urine organic acid analysis using GC-MS

Three drops of 6N HCl were added to 1 ml of urine or of 80 mg/100 ml control substrates (Table 1). NaCl was added until saturated. Then, 1 ml of ethylacetate, as a solvent to extract OA, was added. After the solution was mixed and centrifuged at 3,000 rpm for 3 minutes, the upper layer was transferred to a new tube and evaporated with nitrogen gas from N-evaporator till dry. We repeated extraction of organic acids two more times, each with 1 ml of ethylacetate. When it dried, BSTFA-TMCS [(N,O-bis (trimethylsilyl) trifluoroacetamide-trimethylchlorosilane) (Supelco, PA, USA)] 100  $\mu$ l was added, then mixed, and heated at 90°C

Table 1  
Standards used and their retention times.

No	Standards	Retention times (min)
1.	Methylmalonic acid	9.50
2.	Adipic acid	19.93
3.	Succinyl acetone	23.31, 24.55, 25.44
4.	Orotic acid	28.57
5.	Sebacic acid	33.22
6.	Undecanedioic acid	36.61

in a water bath for 10 minutes. The sample was then injected into the GC (HP 5890 series II PLUS) using Helium as carrier gas with the flow rate of 0.5 ml/minutes. The column used was HP-Ultra2, 25 m x 0.2 mm x 0.33  $\mu$ m. The injection condition was "split (20:1), inlet at 250°C". The oven temperatures were 100°C for 1 minute, then increased with the rate of 3°C per minute to 250°C and sustained for 1 more minute. The substances were detected by mass selective detector (Hewlett Packard 5972 series) at 280°C and were identified by a library kindly provided by Dr George Thomas of the Kennedy Krieger, USA and Dr Tina Cowan at the University of Maryland, USA.

### Patients

**Family 1:** Patient 1 was born at 37 weeks of gestation. The pregnancy, labor and delivery was unremarkable. His parents were second cousins (see pedigree in Fig 1). The patient's older brother (patient 2) died of hypoglycemia and severe metabolic acidosis at age 5 months. Patient 1 suffered from persistent pulmonary hypertension and pneumonia requiring ventilatory support for the first 16 days of life. Because his older brother was suspected of having an organic acidemia, the patient was given carnitine 300 mg/kg BW/day since the first week of life. At age 3 weeks, his general condition improved and he was discharged from the neonatal intensive care unit. The carnitine was discontinued and he was fed on regular formula. At age 2 months, he developed lethargy. Physical examination revealed mild dehydration, jaundice, and tachypnea. Laboratory data demonstrated pancytopenia with hemoglobin 9.52 g/dl, hematocrit 28.3%, white blood cell count 1,090 cells/mm<sup>3</sup>, and platelet 19,300 /mm<sup>3</sup>. Urine pH was 6 and urine ketone 2+. He did not have hypoglycemia. Serum sodium was 133 mEq/l, potassium 4.4 mEq/l, chloride 94 mEq/l, bicarbonate 17 mEq/l and the anion gap of 22 mEq/l. BUN was 6 mg/dl, Cr 0.5 mg/dl, total bilirubin 6.46 mg/dl, direct bilirubin 5.71 mg/dl, alkaline phosphatase 220, SGOT 69 U/l, SGPT 95 U/l, ionized calcium 1.33 mmol/l (normal range 1.15-1.8 mmol/l), magnesium 2.0 mg/dl (normal range 1.6-2.6 mg/dl), and ammonia 492  $\mu$ g/dl (normal range: 25-94  $\mu$ g/dl). Urine ferric chloride test and DNPH tests were negative. Urine p-nitroaniline test was positive.

**Family 2:** An 8-month-old boy (patient 3) presented with fever, vomiting and lethargy. He was born to a G3P2 27-year-old mother and a 32-year-old unrelated father. The pregnancy and delivery

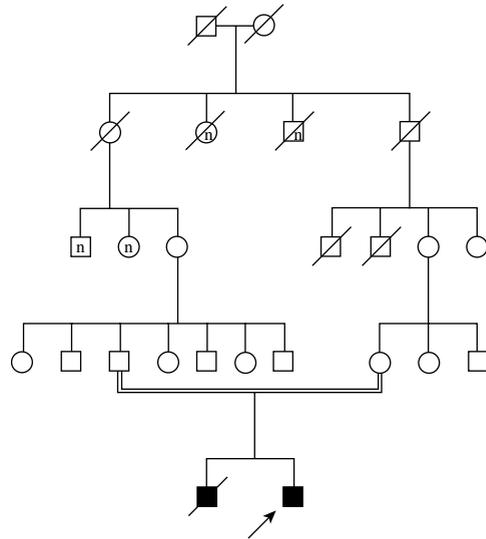


Fig 1—Pedigree of the family 1.

were unremarkable. He had an older brother (patient 4) who died at age 2 years because of severe acidosis. Another older sister had been normal. On physical examination, he was tachypneic, lethargic, and moderately dehydrated. Blood cell counts were within normal limits. Serum sodium was 134 mEq/l, potassium 2.5 mEq/l, chloride 98 mEq/l, bicarbonate 11 mEq/l and the anion gap 25 mEq/l. BUN was 2 mg/dl and Cr 0.6 mg/dl. Liver function tests were unremarkable. Ammonia level was 350  $\mu$ g/dl (normal range: 25-94  $\mu$ g/dl). Urine ferric chloride test and DNPH tests were negative. Urine p-nitroaniline test was positive.

**Family 3:** A one-month-old boy (patient 5) presented with lethargy for 3 days before admission. His parents were second cousins. His older brother (patient 6) died at age 3 months from severe metabolic acidosis without a definite diagnosis. However, the mother recognized the similar manifestations in her 2 children. Upon admission, his body weight was 2,700 g (his birth weight was 3,200 g). Physical examination revealed moderate dehydration and hepatomegaly with palpable liver 2 cm below his right costal margin. Laboratory data showed severe metabolic acidosis with initial bicarbonate of 6 mEq/l. The sodium was 132 mEq/l, potassium 4.8 mEq/l, and chloride 100 mEq/l. The blood sugar was 78 mg/dl, BUN 18 mg/dl, and Cr 0.8 mg/dl. The ammonia level was 600  $\mu$ g/dl. Urine examination showed pH of 5.5, specific gravity 1.026, ketone 2+ and negative for protein and sugar.

**Family 4:** A 3-year-old girl (patient 7) presented with upper respiratory tract infection. She was born at term following an uncomplicated pregnancy, labor, and delivery. She was admitted once at age 8 months due to respiratory tract infection. Her parents were not consanguineous. Her brother (patient 8) had died of severe metabolic acidosis at age 1 year and 2 months. Physical examination of patient 7 revealed moderate dehydration and tachypnea. Her blood counts were within normal limits. Urine pH was 5 and urine ketone was 4+. She had severe metabolic acidosis with serum sodium of 139 mEq/l, potassium 4.6 mEq/l, chloride 109 mEq/l, bicarbonate 2 mEq/l and the anion gap 28 mEq/l. Her blood sugar was 77 mg/dl, BUN 8.21 mg/dl, Cr 0.54 mg/dl, ammonia 86  $\mu$ M (normal range: 9-33  $\mu$ M), and lactate 5.8 mM (normal range: 0.89-2.09 mM). Urine ferric chloride test and urine reducing substance test were negative. Plasma and urine amino acid analyses were unremarkable.

## RESULTS

### Urine organic acid analysis

All substances used as controls were retrieved and correctly identified by the libraries. Table 1 illustrated retention times for each substance. Fig 2 (A-E) demonstrate tracing of the substances.

### Patients

Urine organic acid analysis of the patient 1 from family 1 (Fig 3A) revealed large amounts of 3-hydroxypropionate and methylcitrate. Small peak of 3-OH isovalerate was present. The pattern was consistent with propionic acidemia. Urine samples of patient 3 from family 2 (Fig 3B) and patient 5 from family 3 (Fig 3C) revealed huge peaks of methylmalonic acid, which was diagnostic for methylmalonic acidemia. Urine organic analysis of the patient 7 from family 4 (Fig 3D) revealed large amounts of 3-methylcrotonylglycine and 3-hydroxyisovalerate. The pattern was consistent with 3-methylcrotonyl CoA carboxylase deficiency.

## DISCUSSION

In 1966, isovaleric acidemia, the first organic acidopathy was described. Since then, more than 50 phenotypically different organic acidemias are

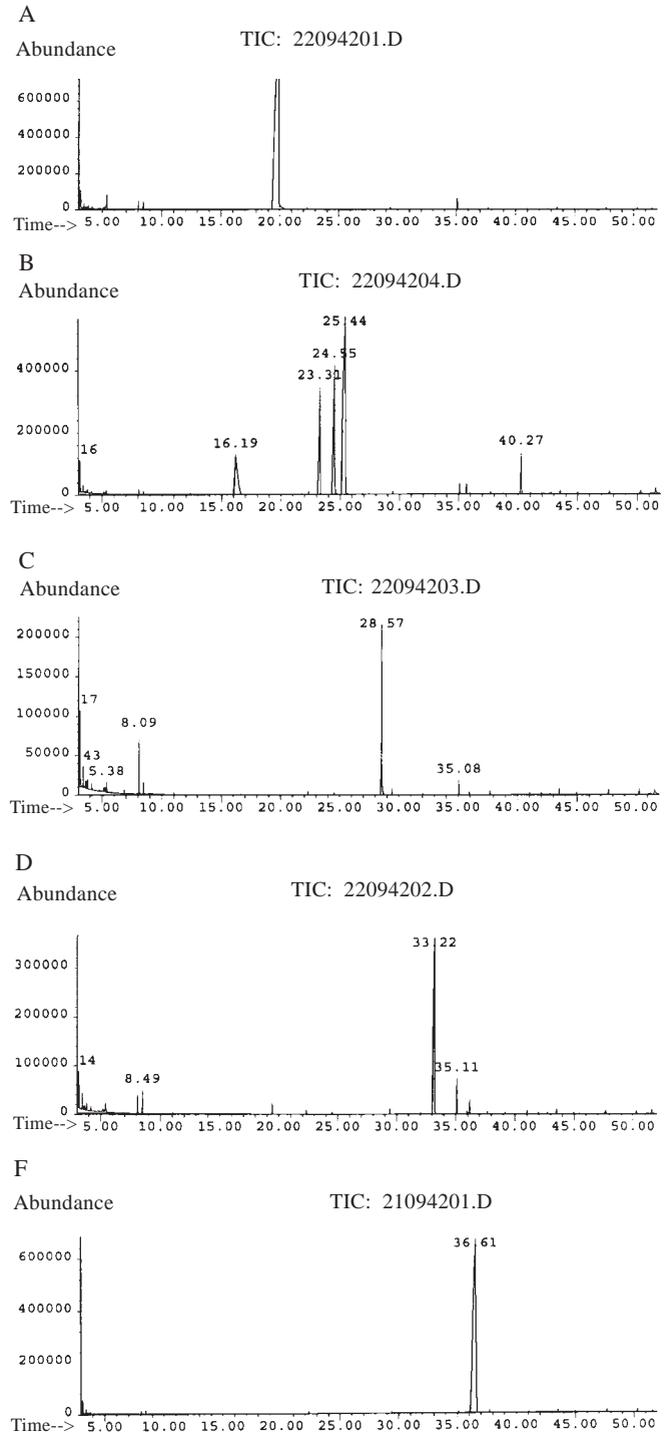


Fig 2—tracings of adipic acid (A), succinyl acetone (B), orotic acid (C), sebacic acid (D), and undecanedioic acid (E) as internal standard.

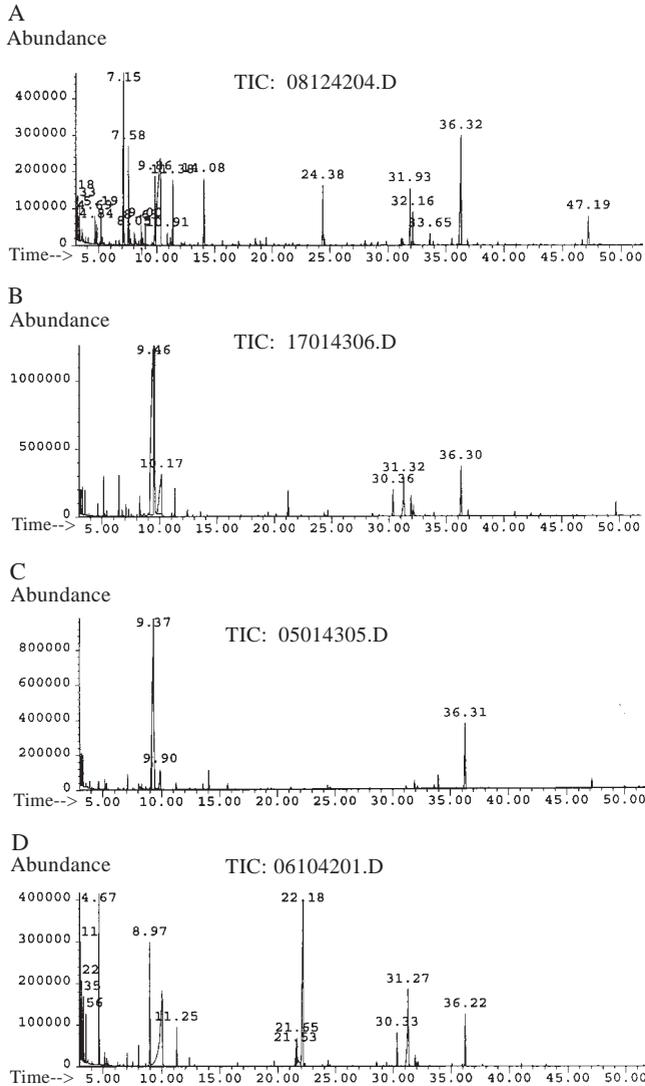


Fig 3—Urine organic acid tracing of patient 1 (A), patient 3 (B), patient 5 (C), and patient 7 (D). Panel A showed large peaks of 3-hydroxypropionate at 7.15 minutes and methylcitrate at 31.93 and 32.16 minutes. A small peak of 3-OH isovalerate was present at 9.00 minutes. The peak at 36.32 minutes was the undecanedioic acid added as an internal standard. The pattern was consistent with propionic acidemia. Panel B and C revealed huge peaks of methylmalonic acid at 9.46 and 9.37 minutes, respectively. The peaks at 36.30 minutes in panel B and at 36.31 minutes in panel C were the internal standard. They are diagnostic for methylmalonic acidemia. Panel D revealed large peaks of 3-methylcrotonylglycine at 21.65 and 22.18 minutes and 3-hydroxyisovalerate at 8.97 minutes. The peak at 36.22 minutes was the internal standard. The pattern was consistent with 3-methylcrotonyl CoA carboxylase deficiency.

Table 2

Indications for urine OA analysis.

1. Acute, chronic or recurrent metabolic acidosis, with or without an anion gap, hypoglycemia or hyperammonemia especially when induced by protein intake or infection.
2. Episodic neutropenia, and thrombocytopenia when associated with ketoacidosis.
3. Unusual odor.
4. Childhood onset of progressive extrapyramidal disease.
5. Reye syndrome when recurrent, familial, or in infancy.
6. Neurologic syndrome with alopecia and rash.

identified (Ozand, 1991). Several symptoms suggest OA and some metabolic screening tests are helpful (Buist, 1995). Table 2 illustrates some indications for urine OA analysis with GC-MS.

In developed countries, GC-MS is the most common method used to diagnose the disorders. Although GC-MS has long been available in Thailand, it has been mainly used to identify medications such as anticonvulsants, and illicit drugs, for examples, heroin. Unfortunately, it had never been utilized to diagnose OA. In collaboration between Department of Pediatrics and Department of Forensic Medicine of King Chulalongkorn Memorial Hospital, we modified a method currently used at the Kennedy Krieger Institute at Baltimore, MD, USA for diagnosing OA in Thailand. The important steps are isolation of the organic acids from physiological fluids, formation of volatile derivatives, and GC-MS analysis. Isolation of the acids is commonly accomplished by solvent extraction, which is ethylacetate in this case. Volatile trimethylsilyl (TMS) derivatives are the most useful and versatile for the wide range of chemical groups in organic acids. They formed by heating with bis-trifluoroacetamide (BSTFA). The capillary columns have an excellent capacity to handle the wide range of acid concentrations. Then, unambiguous identification of compound is made by mass spectra.

Collection of urine specimens from patients has to be careful. The urine should be collected during the metabolic derangement. Urine during normal periods usually provides no abnormal acids and gives a false negative result. Urine should be collected in containers without preservatives and

frozen as soon as possible. Then the samples can be stored at -20°C until analyzed. Making a diagnosis of an organic acidemia is by identification of abnormal organic acids not present in urine of normal individuals. Therefore, even this method is a qualitative assay, it is very powerful and has few problems in making diagnosis.

Previously, there were few reported cases of organic acidemias in Thai patients (Wasant, 1995). In addition, they were diagnosed either by metabolic screening tests performed in Thailand or by GC-MS performed in developed countries. Here, using our newly developed technique, we were able to identify 4 more families with organic acidemias. Two had methylmalonic acidemia, one had propionic acidemia, and the other had 3-methylcrotonyl CoA carboxylase deficiency.

Each of our 4 families had 2 affected siblings. Even though none of the urine samples of the first child in each family were available and analyzed, we believe they had the same disorders as their younger siblings because of their similar clinical and laboratory data. Two of our four families had history of consanguineous marriage emphasizing the autosomal recessive pattern of inheritance in these metabolic disorders.

Availability of a laboratory in Thailand and affordability of the test are expected to result in earlier diagnosis and identification of more cases of OA. Therefore prompt and proper treatment can be anticipated which should lead to better prognosis for patients with this group of disorder.

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