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Inborn errors of metabolism discovered in Asian department of pediatrics and mental retardation research center

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Abstract

To heighten the effectiveness of chemical diagnosis for inborn errors of metabolism (IEM) using urease pretreatment and GC–MS analysis, a sample collection and transportation method was contrived. The resulting ''filter paper set'' allows simple urine collection and transportation, and enables anyone from anywhere to receive the GC–MS analysis without the limitations of place or time. Using filter paper sets, high-risk screening of undiagnosed children or mentally retarded children with unknown cause was conducted in cooperation with hospitals and universities in several Asian countries. During 8 months 203 patients from China and India were analyzed and 20 cases of IEM were chemically diagnosed. These diagnoses greatly contributed to the treatment of children with intractable diseases who lived in Asian countries where analytical techniques and facilities for IEM were not sufficient.

Keywords: Inborn errors of metabolism; GC–MS

1. Introduction

In 1908, Garrod [1] introduced the concept of ''inborn errors of metabolism'' based on his research work on metabolic diseases such as alkaptonuria. An ''inborn error of metabolism'' is defined as a disease that exhibits clinical symptoms due to a birth defect which blocks a metabolic pathway. This is an error in genetic code that is caused by alteration of, or deletion or insertion in, the base sequence of DNA in genes at the time of fertilization, resulting in lowered or deficient activity of an enzyme. Due to the fact that the metabolic pathway of a deficient enzyme is blocked in these diseases, its metabolic substrates are accumulated or its metabolites are reduced. Therefore, it is possible to identify the blocked pathway and the deficient enzyme from the presence of abnormally increased or decreased compounds.

The concept of ''inborn errors of metabolism'' introduced by Garrod was proven when Cori and Cori [2] discovered an enzyme deficiency of glucose-6-phosphatase in von Gierke's disease in 1952.

In the 1940s, chromatography was developed by Consden et al. [3] and was applied to the study of inborn errors of metabolism, especially errors of amino acid metabolism. The bacterial inhibition assay (BIA method) developed by Guthrie [4] in 1961 played an important part in an application to newborn mass screening of inborn errors of metabolism. This method was used for phenylketonuria

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(PKU) at first, and was also applied to maple syrup urine disease (MSUD), histidinemia, etc. In 1966 Beautler et al. [5] established an enzyme assay using dried blood spots on filter paper and applied this to screening of galactosemia. Also, Dassault [6] made mass screening of infantile hypothyroidism possible by combining chromatography and radio-immunoassay (RIA method). As new analytical methods were developed, multiple screening of inborn errors of metabolism, such as PKU, MSUD, homocystinuria, galactosemia and so on, became popular throughout the world. Presently, semi-quantitative methods such as colorimetry, paper chromatography, thin-layer chromatography, BIA, and Beautler's method, RIA, enzyme immunoassay (EIA), etc. are used. Very recently, more accurate analytical methods such as liquid chromatography, gas chromatography, and nuclear magnetic resonance (NMR) have been introduced.

Gas chromatography mass spectrometry (GC–MS) has been used for analyzing organic acids in urine for chemical diagnosis of metabolic disorders of organic acids since 1966 because it assures high accuracy and sensitivity as well as provides information about multiple compounds simultaneously. Since Tanaka [7] discovered iso-valeric acidemia using GC–MS in 1966, many metabolic disorders of organic acids were found [8–13], and the usefulness of GC–MS was widely approved [14–17]. Since 1992, Matsumoto et al. improved the sample pretreatment originally developed by Shoemaker et al. [18], and named it ''urease pretreatment'' [19–22]. This urease pretreatment enabled simultaneous analysis of organic acids, amino acids, sugars, sugar-alcohols and nucleic acid bases for these metabolic disorders, which enlarged the application range of GC–MS to inborn errors of metabolism [23,24]. At present, GC–MS is an indispensable method for diagnosing inborn errors of metabolism, and is widely recognized for its effectiveness in related fields.

The authors contrived a method for sample collection and transportation to heighten this effectiveness, and developed a urine filter paper set which allowed anyone who lived anywhere to receive the GC–MS analysis for inborn errors of metabolism in cooperation with several hospitals and universities located all over the world [25,26]. The authors conducted high-risk screening of undiagnosed children at the department of ICU and neurology in China, and undiagnosed children with mental retardation who underwent rehabilitation at the Center for Research in Mental Retardation in India. Some results are reported in this paper.

2. Experimental

2.1. Urine filter paper set

A urine filter paper set consists of filter paper for urine collection and a drying bag (called a ''set bag'') that allows wet filter paper to dry during mailing. The filter paper is 2 cm×3 cm and made of Toyo Roshi No. 2 that can contain a sufficient amount of urine for GC–MS analysis. The filter paper part is covered with non-woven film to prevent tearing. The drying bag is equipped with a sealing device and is filled with desiccant that can dry over 90% of 3 ml urine contained in a filter paper within 8 h. To collect urine, the filter paper (covered with non-woven film) should be soaked in a urine sample, drained well, placed in the set bag, and then sealed. The urine filter paper set can be sent to the analytical institute by air-mail.

2.2. Subjects

Urine samples were collected from 79 mentally retarded children who underwent rehabilitation at the Centre for Research in Mental Retardation in India, and 124 undiagnosed children at the department of pediatric neurology in Capital Institute of Pediatrics and department of ICU in First Hospital of Beijing Medical University in China. Then the urine samples were air-mailed to MILS in Japan for the GC–MS analysis.

2.3. Sample treatment

The filter paper part was cut out from the filter paper set, and the non-woven film was removed. The filter paper was folded once, and was placed in the outer tube of a 2.5-ml disposal injector that was set on the spit tube. 1.5 ml of distilled water was slowly poured on top of the filter paper, left for 5 min, and

then centrifuged at 2000 rpm for 5 min to obtain urinary eluate.

0.1 ml of eluate was treated in the same way as fresh urine. That is, the eluate (0.1 ml) was incubated with 20 μl urease solution (containing 15 U of enzyme) at 37°C for 15 min. n-Heptadecanoate (Tokyo Kasei) 20 μg was added as an internal standard, and the eluate was deprotenized with 1 ml of ethanol by centrifugation. After removing precipitate, it was evaporated to dryness, and the residue was trimethylsilylated with 100 μl of N,O-bis-trimethylsilyltrifluoroacetamide (BSTFA) and 10 μl of trimethylchlorosilane (TMCS) at 90°C for 40 min. 1 μl of the derivatized extract was injected into GC–MS.

2.4. GC–MS analysis

All analyses were done on a Shimadzu GC–MS QP-5050A using helium gas as a carrier gas with a velocity of 24 ml/min to an ultra alloy-5 silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Frontier Lab, KK). The temperature was raised from 60°C to 325°C at 17°C/min. Each 1-μl sample was automatically injected in 20:1 split mode and mass spectrum was scanned by the electron impact (EI) method in 0.25 s low resolution mode from m/z 50 to m/z 650.

2.5. Data analysis

Peaks in the TIC chromatogram that showed the profile of urinary metabolites were identified from each mass spectrum. Abnormal excretion of marker compounds for 101 metabolic diseases, including 52 amino acidopaties and organic acidemias, 8 lactic acidemia and hyperpyruvic acidemia, 1 disease of TCA cycle abnormality, disorders of 3 fatty acid metabolism, 5 peroxisomal metabolism, 9 purine and pyrimidine metabolism, 9 sugar metabolism and 14 other metabolism, were surveyed for screening of inborn errors of metabolism.

3. Results

From Jan. 1, 1999 to August 30, 1999, 203 high-risk samples were analyzed. Among 203 undiagnosed children 20 cases of inborn errors of metabolism were found (Table 1): 2 cases of glutaric aciduria, 1 case of methylmalonic acidemia, 2 cases of Canavan disease, 1 case of alkaptonuria, 4 cases of galactosemia, 4 cases of PKU, 2 cases of ornithine carbamoyltransferase deficiency (OCTD), 1 case of pyroglutamic aciduria, 1 case of lysinuria and 2 cases of ketotic dicarboxylic aciduira.

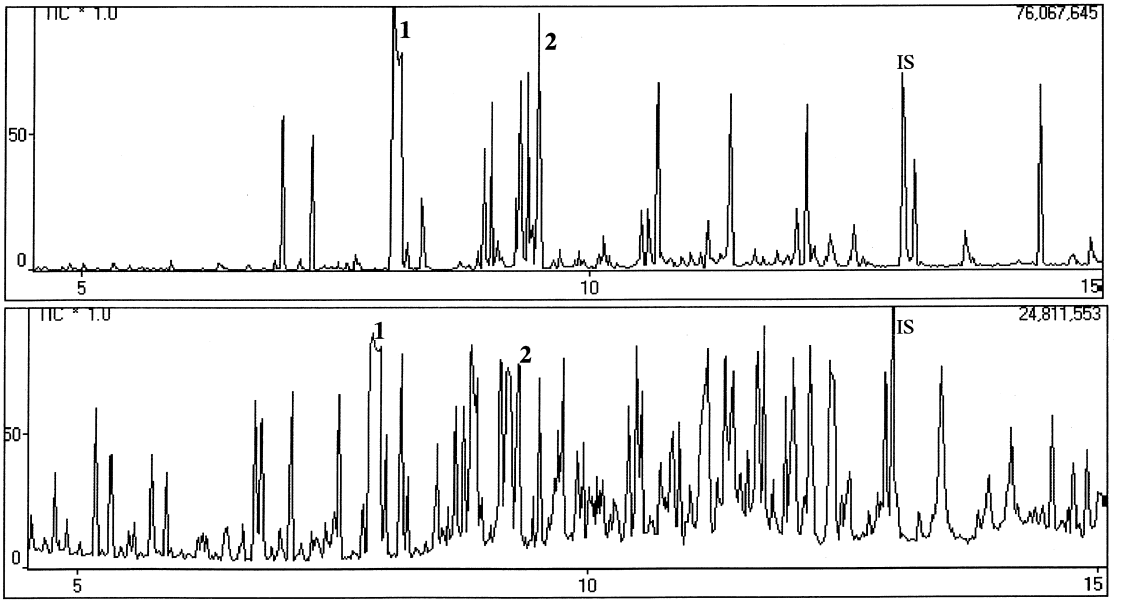
Table 1
List of 20 cases chemically diagnosed by GS/MS

Disease	Patient	Age	Sex	Country
Gluataric aciduria	R.D	2 y	M	India
Gluataric aciduria	W.Y	6 m	M	China
Methylmalonic acidemia	L.H	8 m	M	China
Canavan disease	S.J	1 y	M	India
Canavan disease	H.A	4 m	M	India
Alkaptonuria	S.D	2 y	M	India
Galactosemia	K.V	2 m	M	India
Galactosemia	Z.D	2 m	M	China
Galactosemia	L.Z	7 m	M	China
Galactosemia	Z.K	9 m	M	China
PKU	L.F	1 y	M	China
PKU	Y.W	1 y	F	China
PKU	Z.H	7 m	M	China
PKU	R.Q	2 y	M	China
OTC	S.J	10 y	F	China
OTC	S.J	37 y	F	China
Pyroglutamic aciduria	L.P	12 y	F	China
Lysinuria	L.H	6 m	M	China
Ketotic dicarboxylic aciduria	Z.P	14 y	M	India
Ketotic dicarboxylic aciduria	H.Z	11 y	F	China

Case R.D. a 2-year-old, male: he had febrile convulsions when 4 months old and 6 months old and was hospitalized for 10 days each. After that, convulsions reoccurred, and mental retardation was observed though there was no impediment in vision or hearing. He was undergoing rehabilitation at CREMERE. The TIC chromatogram of urinary metabolites showed marked increases in glutaric acid and 3-hydroxyglutaric acid as shown in Fig. 1, and he was diagnosed as having glutaric aciduria [8,13,16,27]. Case W.Y. (6 months, male) from China was also diagnosed as glutaric aciduria.

Case L.H. 8 months, male: he has repeated vomiting and convulsions, and showed a developmental retardation. His TIC chromatogram is shown in Fig. 2. He was diagnosed with methylmalonic acidemia [8,28].

Fig. 3 shows TIC Chromatograms of a 10-year-old



* 1: glutaric acid, 2: 3-hydroxyglutaric acid

Fig. 1. TIC chromatograms of urinary metabolites from patients with glutaric aciduria (Case R.D., India and Case W.Y., China).

girl and her mother. The girl repeatedly suffered from headaches, vomiting, convulsions, and coma, and was diagnosed with virus encephalitis by a local hospital; however, there was no corresponding observations found from CT and MRI tests. After she was discharged from the hospital following some improvement, she had a convulsion again and was admitted to ICU of Beijing Medical University Hospital. When admitted, no abnormality was detected with the laboratory tests except that ammonia concentration in blood was elevated to 183 μmol/l (healthy control: 60 μmol/l or less). The results of GC–MS analysis revealed markedly increased excretion of uracil and orotic acid, suggesting that she suffered from OCTD [29–31]. Her mother's urine was also analyzed, revealing that she also suffered from OCTD.

Case H.H.A.A. 4 months, male: he showed developmental retardation, single cafe-au-lait spot on the face, and mental retardation. GC–MS analysis

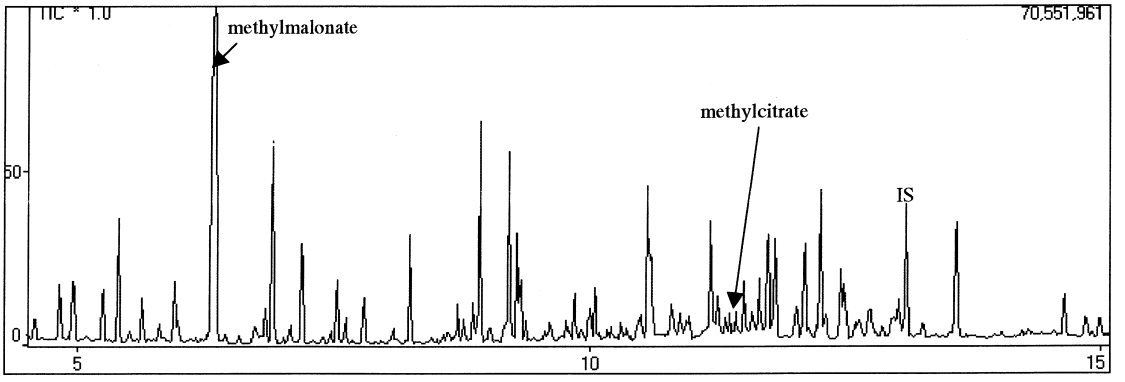
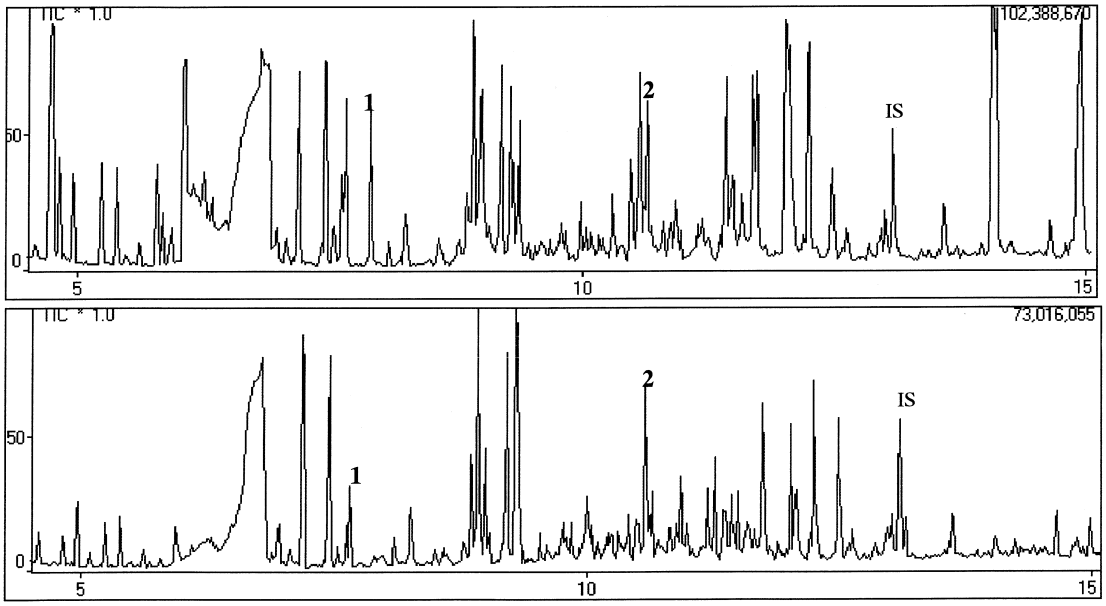


Fig. 2. TIC chromatogram of urinary metabolites from a patient with methylmalonic acidemia (Case L.H., China).



* 1: uracil, 2: orotic acid

Fig. 3. TIC chromatograms of urinary metabolites of OCTD patients (Upper: Case S.J., Lower: Her mother, China).

(Fig. 4) revealed that N-acetylaspartic acid excretion was markedly increased, and he was diagnosed with Canavan disease [32–34]. The CT test, conducted at the same time as GC–MS analysis, suggested mild ventricular megaly and parietal white matter attenuation, which verified Canavan disease.

Figs. 5–8 show the TIC chromatograms from cases of alkaptonuria [35] (2-year-old, male), galactosemia [36] (9 months, female), PKU [37] (1-year-old, male), pyroglutamic aciduria [38] (12-year-old, female), lysinuria [39] (6 months, male), respectively (Fig. 9)

4. Discussion

In some Asian countries, inborn errors of metabolism have not been well recognized, and diagnosis and treatment are not performed sufficiently. The Guthrie test for PKU and infantile hypothyroidism

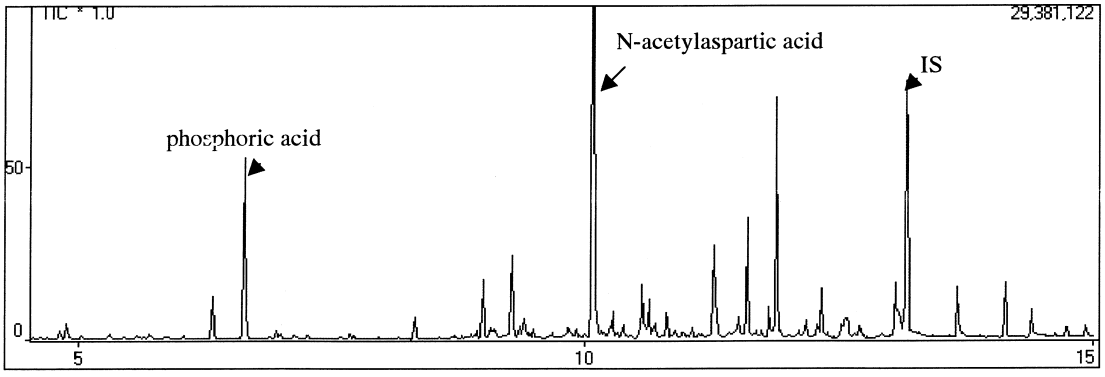


Fig. 4. TIC chromatogram of urinary metabolites from a patient with Canavan disease (Case H.H.A.A., India).

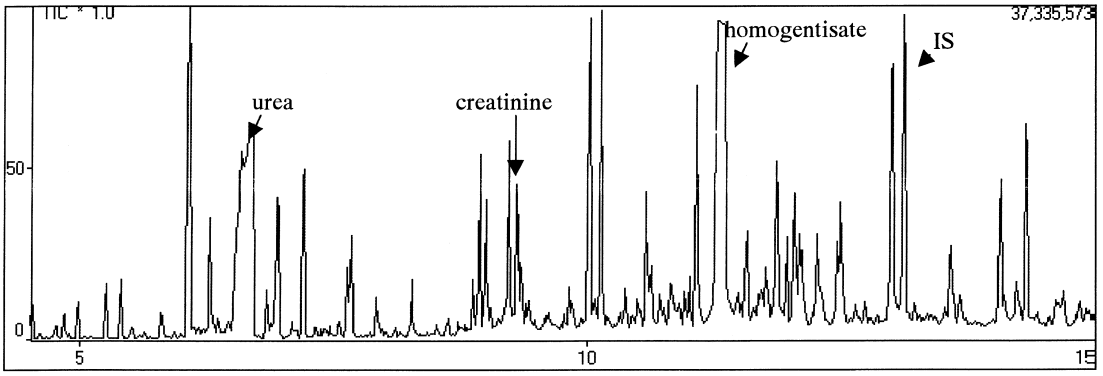
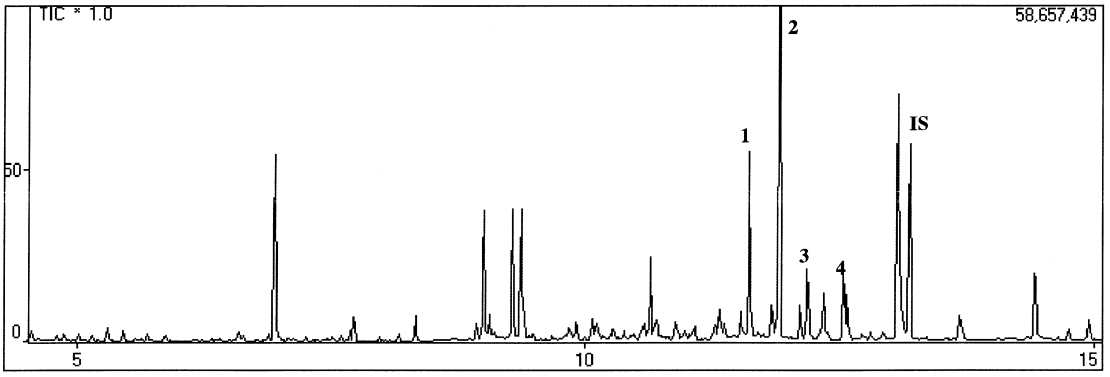
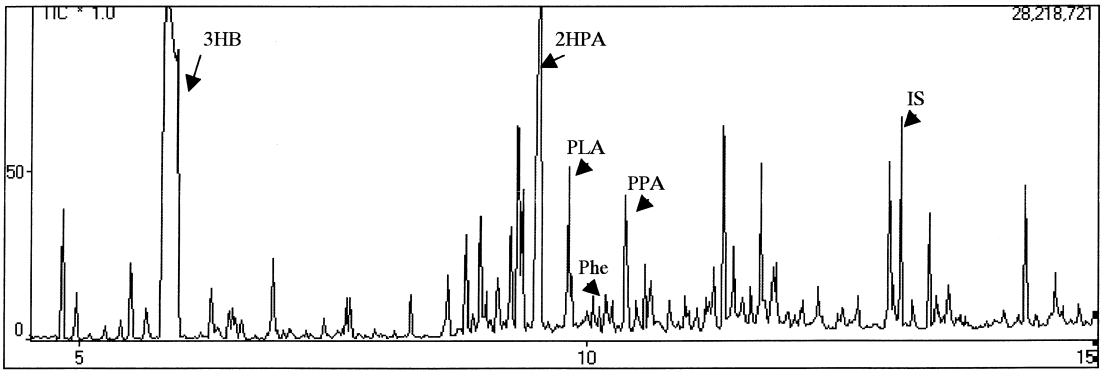


Fig. 5. TIC chromatogram of urinary metabolites from a patient with alkaptonuria (2 y, male).



1: α-galactose, 2: β-galactose, 3: galactitol, 4: galactonate

Fig. 6. TIC chromatogram of urinary metabolites from a patient with galactosemia (9 m, female).



*3HB:3hydroxybutrric acid, 2HPA: 2-hydroxyphenylacetic acid, PLA: phenyllactic acid, Phe: phenylalanine, PPA: phenylpyruvic acid.

Fig. 7. TIC chromatogram of urinary metabolites from a patient with PKU (1 y, male).

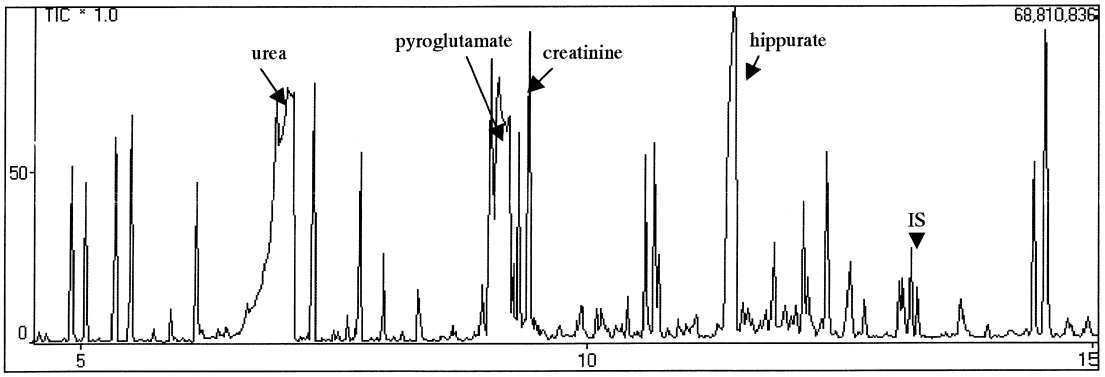


Fig. 8. TIC chromatogram of urinary metabolites from a patient with pyroglutamic aciduria (12 y, female).

has been started in small parts of some countries [39], but it will take considerable time before this test becomes popular in these countries. There are surely a great number of neonates or infants who die from inborn errors of metabolism because they unfortunately do not have a chance to be diagnosed correctly. Even if the patient is lucky enough to be diagnosed and saved from death at an early stage, insufficient treatment may lead to repeated onsets, long-term hospitalization, and may result in mental retardation.

The chemical diagnostic method for inborn errors of metabolism using urease pre-treatment and GC–MS analysis uses multiple compounds as markers, while only one target compound is used for the Guthrie test; therefore, the former diagnosis is more accurate, and can detect more metabolic disorders of amino acids, sugars, sugar-alcohols, nucleic acid bases and nucleosides, as well as organic acids, compared with other diagnostic methods currently used. Also, this method uses urine as sample; urine can be collected easily without invasion which means that this method can be readily adopted in examination centers or for screening. It is, however, regrettable that at present this excellent method is only available in developed countries.

By means of the urine filter paper set the authors developed, chemical diagnosis of inborn errors of metabolism can be performed for children who live in developing countries where the same level of diagnosis is not locally possible. The authors conducted high-risk screening of undiagnosed children and refractory cases from Capital Pediatric Institute, Beijing Medical University Hospital and CREMERE, and 20 cases of inborn errors of metabolism were found among 203 cases. Included are glutaric

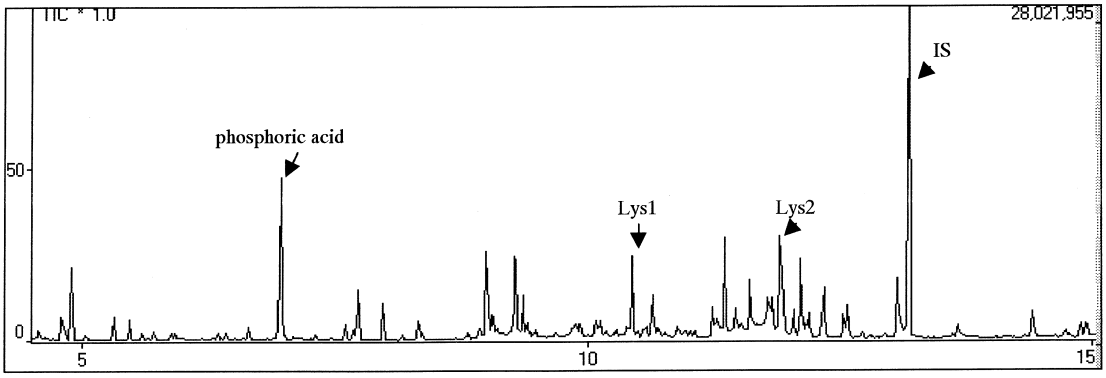


Fig. 9. TIC chromatogram of urinary metabolites from a patient with lysinuria (6 m male).

aciduria and methylmalonic aciduria (organic acid metabolism), PKU and OCTD (amino acid metabolism), galactosemia (sugar metabolism), Canavan disease, alkaptonuria, pyroglutamic aciduria and lysinuria. The results demonstrated the significance of multiple compound analysis by one single GC–MS measurement for diagnoses of a number of metabolic disorders.

These 20 cases were diagnosed in only 8 months, which immediately reflected to the treatment of the patients, and thus a high reputation was obtained. For example, the OCTD patient from China had suffered from chronic headaches, vomiting and convulsions, resulting in mental retardation, and she was repeatedly hospitalized. Her family's financial burden was great. Since the diagnosis was made and her mother was also found to be an OCTD patient, the doctor could explain about her disease, and provide treatment for stopping the further advance of mental retardation. The patient, her family, the doctor and the hospital all regarded chemical diagnosis by GC–MS highly.

Chemical diagnosis in combination with urine collection and transportation by filter paper sets, urease pretreatment and GC–MS analysis is proven to be essential for high-risk screening and diagnosis of undiagnosed or refractory children who live in developing Asian countries. The authors aim to propagate this method widely in other Asian countries to save children who, unfortunately, are undiagnosed or refractory because of the locality.

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