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PILOT STUDY ON NEONATAL MASS SCREENING FOR INBORN ERRORS OF METABOLISM BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY: EIGHTEEN MONTHS EXPERIENCE IN SHIMANE AREA

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We have performed a pilot study of neonatal mass screening for inborn errors of metabolism (IEM) by gas chromatography-mass spectrometry (GC / MS) using urine specimens in the Shimane area. In this project, 1,686 babies born in several institutes in Shimane were screened for 18 months from May, 1996 to October, 1997. The automated metabolic profiling and disease detection system developed by us was applied to this screening. Patients with definite IEM were not identified, but several transient abnormalities in the neonatal period, such as elevation of 4-hydroxyphenyllactate, galactose, or 3-hydroxy-3-methylglutarate, were observed in 112 (6.2%), in particular, more frequently in low birth weight infants. In order to test the usefulness of our screening system, samples from 13 patients with 9 different metabolic disorders previously diagnosed were analyzed. Consequently, methylmalonic acidemia, isovaleric acidemia and glyceroluria were detectable without fail. In cases of propionic acidemia, phenylketonuria, maple syrup urine disease, ornitine transcarbamylase deficiency or glutaric aciduria type 2, the detection of their diagnostic marker metabolites seemed unstable.

Key words: neonatal mass screenning /inborn errors of metabolism / organic acidemia / gas chromatography-mass spectrometry

In Japan, neonatal mass screening for 6 kinds of inborn errors of metabolism (IEM), using dried blood filter paper by simple methods such as Guthrie test, has been used for the past 20 years. The nation-wide mass screening in Japan has produced good results in prevention of handicaps in children. In the current system, however, the number of diseases screened is limited to only six, including phenylketonuria (PKU), maple syrup urine disease (MSUD), homocystinuria, galactosemia, cretinism and congenital adrenal hyperplasia.

Organic acidemia is due to defects in intermediate metabolic steps of amino acids, lipids, or carbohydrates, and can be detected by gas chromatography-mass spectrometry (GC/MS) analysis of urinary organic acids (1,2). Patients with such disorders often present with acute clinical symptoms, such as sucking difficulty, hypotonia, tachypnea, ketoacidosis, or hypoglycemia, early in infancy. Nevertheless, the early detection and intervention of such diseases can often prevent neurological handicaps.

As GC/MS requires some expertise on the equipments as well as the data interpretation, it has not been so widely used in clinical laboratory. Recently, inexpensive, simple and convenient GC/MS equipments have been developed, with the assistance of personal computers and softwares. Furthermore, simple methods for sample preparation have been reported (3,4). Neonatal mass screening is now considered and attempted in several institutes in North America or Europe. The Japanese Society of Biomedical Mass Spectrometry and Neonatal Mass Screening has urged the local pilot study of organic acidemia screening using GC/MS to four institutes of Japan, including Kanazawa Medical University, Kurume University, Chiba Children's Hospital and our Shimane Medical University, all of which were involved earlier in the studies of IEM using GC/MS in Japan.

Hence, we have performed a pilot study of neonatal mass screening on 1,686 babies in Shimane, with the assistance of an automated data processing system developed by us. The usefulness of our data processing system in this screening was tested by analysis of urine specimens from patients previously diagnosed. We report here our 18-month experience with the neonatal mass screening for IEM, in particular, organic acidemias using GC/MS, in the Shimane area.

MATERIALS AND METHODS

Institutes enrolled in this project and collection of samples

The pilot study was started in four areas of Japan: Kanazawa, Kurume, Chiba and Shimane. In the Shimane area, five hospitals shown below were enrolled in this project. The pilot screening was started in May, 1996, and the Matsue Red-cross Hospital, Shimane Medical University and Nita Hospital took part in this study. In April, 1997, the Gotsu-saiseikai Hospital and Masuda

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Red-cross Hospital joined in place of the Nita Hospital. The data were taken for 18 months, between May, 1996 and October, 1997. During the period, urine specimens from 1,686 newborn infants were screened. The specimens were collected 3 to 6 days after birth using the neonatal urine collector bags, stored in refrigerators at 4℃, and then transferred basically once a week to our laboratory in the Department of Pediatrics, Shimane Medical University.

Reagents

Margarate (MGA) was purchased from Wako Chemical, Kyoto (Japan); even-numbered hydrocarbon mixture (C10 to C26) and tetracosane (C24) from Seikagaku-Kogyo Co., LTD, Tokyo (Japan); and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Nakarai-Tec Co., LTD, Tokyo. Urease was purchased from Sigma (St. Louis, MO).

Preparation of Samples

Creatinine concentration was first measured by the Jaffe's method with UV-1600 UV-visible spectrophotometer, and 100μl of urine sample was taken for the screening. Samples were prepared according to the method of Matsumoto and Kuhara (4). Briefly, 10 U of urease was added to 100μl of urine to degrade urea in urine, and the mixture was incubated at 37℃ for 30 minutes. The sample was deproteinized with 900μl of ethanol and centrifuged at 10,000 rpm for 5 minutes. As internal standards, 20μg each of magarate and tetracosane were added to the supernatant. The supernatant was dried up completely in a vacuum evaporator. Then the residues were trimthylsilated with 100μl of BSTFA and 10μl of TMCS at 80℃ for 30 minutes. One μl of the resultant solution was applied to GC/MS in the split mode (10:1).

GC/MS analysis

GC/MS equipment used was Shimadzu QP-5000. The GC column was DB-5 capillary column (30 m×0.25 mm i.d., 0.25μm film thickness). After an initial holding at 100℃ for 1 minute, the temperature was programmed at a rising rate of 10℃/min up to 290℃ where it was held for 8 minutes. The temperature of the injection port and transfer line was 280℃ each.

Samples from patients previously diagnosed

To test the screening system of GC/MS analysis and evaluate the reliability of the diagnostic algorithms developed by us (5), urine samples from 13 neonatal patients with 9 different metabolic disorders diagnosed previously were analyzed. The samples had been stored at −30℃ until analysis and 100μl of specimens were taken. The number of patients and the disorders are as follows: 3 of methylmalonic acidemia; 3 of propionic acidemia; 2 of isovaleric acidemia; one each of glutaric aciduria type 2 (GA2), glyceroluria, PKU, MSUD and ornitine transcarbamylase deficiency(OTCD).

Automated metabolic profiling and disease detection system

The automated GC/MS data processing system we developed for the diagnosis of organic acidemias was employed in this pilot screening. The principle of the system was described elsewhere (5,6). In this program, methylene unit (MU) value, quantification ion (Q ion), confirmation ion (C ion), ratio of their peak intensities (C/Q) were required. Since we already compiled the data on organic acids, we determined the necessary data on amino acids, carbohydrates or others and added them to the previous data. Hence, in this study, 140 compounds including organic acids, amino acids and carbohydrates or others were searched and quantified for each metabolite.

Then, a normal table including mean values, standard deviations and ranges for each compound was made by analysis of urine samples from 30 normal neonates. The fixed value was expressed by peak relative area (PRA)%/mg creatinine. The mean value plus 5 SDs was set as the upper cutoff. Compounds whose value, PRA%/mg creatinine, was over the cutoff levels were marked with asterisks as abnormal metabolites suspected. In this program, furthermore, disease names were suggested from the combination of abnormal compounds detected. Twenty-two metabolic diseases as described in the literature (4) were enrolled in this program.

RESULTS

Results of the pilot screening in Shimane area

The results of the 18-month pilot study performed in the Shimane area were shown in Table 1. Although patients with definite IEM were not identified, several transient abnormalities during the neonatal period were observed. The incidence was 6.2% (112 of 1686 infants screened), including 48 cases of tyrosyluria (increased excretion of 4-hydroxyphenyllactate) (2.8%), 29 of ele-

Table 1. Results of the pilot study of neonatal screening in Shimane area for 18 months(May,1996-October,1997)

		Birth weight (g)			
	total	~1499	1500 ~2499	2500 ~3999	4000~
Babies screened	1686	33	202	1440	11
Definite inborn errors	0	0	0	0	0
Transient elevation	112	8	23	81	0
(%)	(6.6%)	(24.2%)	(11.4%)	(5.6%)	(0%)
4-OH-phenyllactate	48	4	15	29	0
3-methyl-3-OH-glutarate	29	0	0	29	0
Galactose	17	3	5	9	0
Glycerol	3	1	1	1	0
Methylmalonate	3	0	0	3	0
3-OH-propionate	3	0	1	2	0
Glucose	2	0	0	2	0
Glycerol-3-phospharate	2	0	0	2	0
Uracil	2	0	0	2	0
Threonine	1	0	0	1	0

vated 3-methyl-3-hydroxyglutarate (HMG) (1.7%), 17 of galactosuria (1.0%), 3 each of glyceroluria, and slight increases of methylmalonate or 3-hydroxypropionate, 2 each of elevated excretion of glucose, glycerol-3-phosphate, uracil and one of slight elevation of threonine.

The incidence of the transient abnormalities was higher in the low birth weight infant group as shown in Table 1. Tyrosyluria seemed to be due to tyransient neonatal tyrosinemia because it was transient and without specific symptoms. An elevation of HMG was observed in normal birth weight infants, but the extent was slight and transient. Galactosuria or glucosuria was considered to be related to timing of milk feeding. The Paigen method that is used in the nation-wide screening of Japan revealed no abnormalities in these babies showing galactosuria. Concerning glyceroluria, the amount of glycerol was large in all 3 cases. Two were from babies under the treatment with glycerol against encephalopathy due to neonatal asphyxia. It was possible that infusion of glycerol resulted in glyceroluria in these two cases. The cause of the third case of glyceroluria, however, was unclear, because this case could not be followed up after the discharge from the hospital. Congenital glycerol kinase (GK) deficiency which is inherited in an X-linked fashion, is most famous as a disease that shows massive excretion of glycerol in urine. This infant, however, was female, and showed no special symptoms. It was unlikely that the third case had GK deficiency. Concerning the elevation of other metabolites, such as methylmalonate, 3-hydroxypropionate and so on, the mechanism or pathophysiology was unclear at the present point. However, the extent of increased excretion was small and transient, and the babies showing such abnormality had no special symptoms.

Table 2. Metabolic profiling table of a 2 day-old infant with isovaleric acidemia, from the automatic GC/MS data processing system we developed.

Data File : OIVA1.D14
Comment :

ID	Compound	VALUE		NORMAL	RANGE		FACTOR
2	Glycolic-2	0.3022	(	0.20	0.00 - 1.60)		1.51
4	Glycine-2	1.8793	(	11.10	0.00 - 111.60)		0.17
22	Phosphoric acid	2.8907	(	47.50	0.00 - 613.10)		0.06
26	Succinic-2	0.1079	(	0.70	0.00 - 6.40)		0.15
47	Isovalerylglycine-1	6.8676	* (	0.00	0.00 - 0.00)		?
64	Creatinine-3	0.9213	(	23.20	0.00 - 194.80)		0.04
83	Arabitol-2(3)	0.5286	(	1.20	0.00 - 7.10)		0.44
91	Glucose-2(1)	0.6058	(	0.40	0.00 - 3.30)		1.51
92	Citric-4	2.7201	(	7.60	0.00 - 79.70)		0.36
94	Galactose-2(2)	0.5612	(	0.40	0.00 - 3.30)		1.65
111	Glucose-2(4)	1.5083	(	2.60	0.00 - 24.30)		0.58
112	Galactose-2(6)	0.5559	(	2.40	0.00 - 36.50)		0.23
119	Glucose-2(6)	1.9961	(	3.20	0.20 - 16.20)		0.62
139	Margaric-1(IS1)	3.9448	(	5.20	1.20 - 17.70)		0.76
140	Tetracosane(IS2)	1.8710	(	1.10	0.00 - 4.50)		1.70

No.	Diseases suspected of:
6	isovaleric acidemia

Abbreviation: VALUE, the fixed value, peak relative area (%) /mg creatinine (PRA) on mass chromatogram; NORMAL, mean values of normal controls of neonates; RANGE, ranges of PRA of normal control neonates; FACTOR, times of the mean value. An asterisk close to VALUE means a suspected abnormal metabolite (over mean plus 5 SDs of normal control). At the bottom of the table, a suspected disease name according to the combination of abnormal metabolites, isovaleric acidemia, is shown.

Test of the usefulness of our data processing system for screening

Table 2 and Fig 1 illustrate examples of the data table and total ion chromatogram (TIC), respectively, of a patient with isovaleric acidemia who was diagnosed previously. Table 2 shows the metabolic profiling, putting an asterisk on a compound-47, isovalerylglycine, as an abnormal metabolite suspected. At the bottom of Table 2, "isovaleric acidemia" is printed as a suspected disease for this infant. In Fig 1, the TIC of this case was presented after the data processing. Peaks identified automatically were filled in black and the compound identification number was added at the peak top. A peak judged to be abnormal, peak-47 (isovalerylglycine) was indicated with an asterisk close to the identification number. Hence, we were able to make a final diagnosis, isovaleric acidemia, if necessary, by manual confirmation of its mass spectra.

Table 3 shows the results of the GC/MS analysis in order to test our system on 13 newborn infants with 9 different disorders diagnosed previously. Methylmalonic acidemia, isovaleric acidemia and glyceroluria were well detectable without fail, regardless of the creatinine levels. The amounts of diagnostic markers for such diseases were massive. On the other hand, in the cases of propionic acidemia, PKU, MSUD or OTCD, the amount of each diagnostic marker for the diseases was not so large, sometimes below the detection limit, although we repeatedly tested these samples by our system. In the case of GA2, no abnormality was detected at a few weeks of age of this infant, presumably this case is classified into the late-onset of GA2 (7).

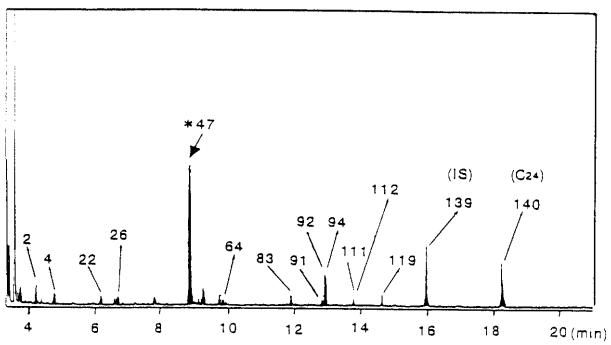

Fig. 1. Total ion chromatogram after automatic data processing. Peaks identified automatically by this program are filled in black with the compound identification number at the peak top. Peaks judged to be abnormal in the program are marked with asterisks. In this chromatogram, peak 47 (isovalerylglycine) was judged to be abnormal. Identification numbers, 139 and 140, represent internal standards, 20 μg each of margarate and tetracosane.

Collection of urine samples from neonates

At the beginning of the pilot screening, a problem of the difficulty in collecting urine from babies had been raised. We examined the success rate of the urine collection by a urine collector bag for 6 months prospectively, dividing institutes into 3 groups, institutes A, B, and C (Table 4). In the institute A, the staffs made every effort to collect as many samples as possible. That is, the staffs tried several times to collect urine samples. In institute B, NICU, urine specimens were collected as routine laboratory tests on admission to NICU. To obtain the urine specimen from patients in NICU was occasionally be difficult because of too small body weight or the emergency. In institute C, sample collection using urine collector bags was done only twice, that is to say, if the first two urine collections were not successful, the staffs gave up the collection. Consequently, the success rate of urine collection, in institutes A, B and C, was 231/233 (99%), 82/111 (70%) and 118/160 (74%), respectively.

Table 3. Results of analysis by our system using urine samples of newborn patients previously diagnosed

Disease (No. of patients)	Creatinine (mg/dl)	Judge	Diagnostic markers detected
Methylmalonic acidemia (3)	18.3	◎	MMA
	20.6	◎	MMA
	9.7	◎	MMA
Propionic aciduria (3)	18.7	◎	3HP, MC
	13.8	○	MC
	10.8	△	3HP
Isovaleric acidemia (2)	57.5	◎	IVG
	27.8	◎	IVG
Glutaric aciduria Type 2 (1)	57.5	×	–
Glyceroluria (1)	15.5	◎	Glycerol
Phenylketonuria (1)	20.6	△	Phe
MSUD (1)	19.1	◎	2HIV, 2HIC, Val
OTCD (1)	5.7	○	Orot

Abbreviations: MMA, methylmalonate 3HP, 3-hydroxypropionate; MC, methycitrate; IVG, isovalerylglycine; Phe, phenylalanine; 2HIV: 2-hydroxyisovalerate; 2HIC, 2-hydroxyisocaprote; Val,valine; Orot, orotate. MSUD, maple syrup urine disease; OTCD, ornitine transcarbamylase deficiency. Judgements from the tests are represented as follows: ◎, excellent in diagnosis, ○, generally good, △, interrogative; ×, difficult to diagnose.

Table 4. The success rate of urine collection from babies using the urine collector bags in 3 institutes

Institute	Collected No. /Total No.	Success rate (%)
A	231/233	99
B	82/117	70
C	118/160	74

This study was carried out for 6 months from July to December, 1996, Institutes A, B and C has the following criteria for urine collection: In A, urine specimens were collected as possible as the staff can; in B, speciments were collected in the connection of routine tests of NICU on admistration, is this group, urine collection is often failed due difficulty in sampling from too small badies or the emergency; in C, urine collection by the urine collector bags was tried only twice.

DISCUSSION

Early detection and intervention of organic acidemias or aminoacidopathies can often prevent the patients from having neurological disturbances. Recently, mass screening of IEM by the mass spectrometric procedures such as urinary organic acid analysis by GC/MS using filter paper(8,9) and acylcarnitine analysis by tandem mass spectrometry(10,11) has been seriously considered and attempted.

The pilot screening has been performed in four areas of Japan where GC/MS analysis is available, since 1996. Till now, 1,686 urine samples have been screened in our area, Shimane. Patients with definite IEM were not found, although in two other institutes of Japan, Kanazawa and Kurume, 3 cases each of IEM, methylmalonic acidemia, glyceroluria and citrullinemia, were already detected in this screening (12). It was reported that the patient with citrullinemia died in the neonatal period and that two other patients had no symptoms at least in infancy. Pathophysiology of such asymptomatic patients remain unknown. The incidence of IEM detected in this screening has been estimated at 1 in about 2,500 to 3,000 cases according to their reports. Although Lehnert estimated the incidence of organic acidemia at about 1/10,000 (14), there is a possibility that the incidence of organic acidemia is larger than expected and that a number of asymptomatic patients exist.

In our experience, there are a considerable number of babies showing transient abnormalities, particularly in low birth weight infants. Further studies on metabolic physiology during the neonatal period, especially in low birth weight infants and on the cutoff levels of metabolites in the neonatal period are needed.

We applied the automated GC/MS data profiling and interpretation system developed by us (5,6) which takes only a minute for data processing of each specimen. It took about 1.5 hours for sample preparation and only 30 minutes for GC/MS analysis. It was confirmed by our experience that a GC/MS equipment is capable of analyzing at least 30 samples a day. Previously, interpretation and determination of the GC/MS data required skill, but the simplified GC/MS analysis combined with specialized personal computers and softwares, like our system will facilitate the mass screening. Organic acid analysis has generally been performed by solvent extraction (1,2), but in this study, we took the direct preparation method because of the simplicity. By this preparation method, amino acids, carbohydrates as well as organic acids can be profiled simultaneously. Therefore, a more comprehensive screening is possible, although this screening is aimed at 22 metabolic diseases at present.

Several problems such as sensitivity or reliability remain to be resolved. Analyzing urine specimens from patients with IEM tested in this study was not reliable in the detection and identification of several diseases such as propionic acidemia, PKU, or MSUD. To determine the amount of samples to be used for screening,

newly-devised analytical methods such as stable isotope dilution analysis, or other derivatization methods, such as t-butyldimethylsilylation are needed. Collection of urine specimens from newborns using urine collector bags was more successful than expected at the beginning of this study. Furthermore, the use of dried urine filter paper should also be considered. We are now attempting at experiments to resolve these problems.

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