Gas chromatographic–mass spectrometric screening for organic acidemias using dried urine filter paper: determination of α-ketoacids

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Abstract

There are several organic acid disorders that require information on α-ketoacids, such as maple syrup urine disease or α-ketoadipic acidemia. The recovery, stability and diagnostic availability of α-ketoacids in dried urine filter paper analyzed by GC–MS with oxime-trimethylsilyl derivatization was studied for organic acidemia screening. The recovery of all nine types of α-ketoacids tested, but for phenylpyruvate, 2-ketoadipate, and *p*-OH-phenylpyruvate, from filter paper samples was acceptable. The stability of pyruvate, branched-chain α-ketoacids, α-ketoadipate and α-ketoglutarate was stable for at least 28 days, although some α-ketoacids such as succinylacetone were unstable. It indicated it was difficult to diagnose only tyrosinemia type 1 among nine specimens from organic acidemia patients tested. The method could be applied to global organic acidemia screening.   © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Organic acidemias are caused by defects in the intermediate catabolic pathways of amino acids, lipids, or carbohydrates. Patients with organic acidemias often have an acute or episodic illness. However, these neonates and young babies can often achieve normal development if detection and intervention are provided early [1]. Hence, neonatal mass screening for organic acidemias has been seriously considered.

Gas chromatography–mass spectrometry (GC–MS) is widely used to identify organic acidemia [2]. Dried urine filter paper (filter paper urine) is a convenient mode for urine collection and transportation from remote places. If it is available for the screening, the organic acidemia screening will be expanded to cases sent from remote places. There are some previous reports on the usefulness of urine filter paper in organic acidemia screening using GC–MS. Chamberlin and Sweeley described urinary organic acid profiles determined by the method of oximation and DEAE column preparation from absorbent filter paper [3]; Tuchman et al. used solvent extraction from urine filter paper, and investigated the stability of metabolites on paper for up to 15 days [4].

Furthermore, a simple and comprehensive preparation method (urease/direct method) was developed for screening organic acidemias, using GC–MS [5,6]. Recently, we reported the practicability of dried urine filter paper for analyzing organic acids,

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amino acids or carbohydrates, using the above urease/direct method and GC–MS [7]. By this method, however, α-ketoacids were not detectable. There are several organic acid disorders that require information on α-ketoacids, including maple syrup urine disease (MSUD), tyrosinemia type 1, lactic and pyruvic (lactic/pyruvic) acidemia or 2-ketoadipic acidemia. To protect α-ketoacids in preparation, the carbonyl group should be converted to oxime-, methoxime- or ethoxime-derivatives [8–10] before the extraction of organic acids.

In the present study, we determined the recovery, reproducibility and stability for 28 days of α-keto-acids on filter paper with preparation of oxime-TMS derivatization and solvent extraction, and analyzed samples from patients previously diagnosed to examine the practicability of the method in detection of organic acidemias.

2. Materials and methods

2.1. Reagents

Pyruvate (Pyr), 2-ketoglutarate (2KG), glutarate (GA) and tropate (TA), hydroxylammonium chloride, ethylacetate, diethylether, N,O-bis-(trimethylsilyl)-fluoroacetamide and trimethylchlorosilane were purchased from Nakarai Tesque (Tokyo, Japan). 2-Ketoisovalerate (2KIV), 2-keto-3-methylvalerate (2K3MV), 2-ketoisocaproate (2KIC), 2-ketocaproate (2KC), phenylpyruvate (PP), succinylacetone (SCA), 2-ketoadipate (2KA), p-OH-phenyllactate (PHPL) and p-OH-phenylpyruvate (PHPP) were from Sigma (St. Louis, MO, USA). The hydrocarbon mixture (C10–C26, even numbers), margarate (MGA) and tetracosane (C24) were from Seikagaku-Kogyo (Tokyo, Japan); 2,2-dimethylsuccinate (DMS) was from Aldrich (USA).

2.2. Preparation of standard solution

The content, selective ions for quantification and conformation (Q-ion, C-ion, respectively) and methylene unit values (MU) for each compound of the standard solution, and internal standards are listed in Table 1. The standard solution contained nine types of α-ketoacids, and two non α-ketoacids, GA and PHPL, these were added for reference purposes.

Table 1
Contents and parameters of compounds in the standard solution and internal standards

Compound	Content, nmol/ml (mg/ml)	Q-ion, m/z	C-ion, m/z	MU
α-Ketoacids				
Pyruvate (Pyr)	0.57 (0.05)	232	247	11.49
2-Ketoisovalerate (2KIV)	1.72 (0.20)	260	232	12.14
2-Keto-3-methylvalerate (2K3MV)	0.77 (0.10)	274	200	12.76
2-Ketoisocaproate (2KIC)	1.54 (0.20)	274	200	12.91
Succinylacetone (SCA)	0.32 (0.10)	212	227	14.53
2-Ketoglutarate (2KG)	0.34 (0.05)	362	377	16.35
Phenylpyruate (PP)	0.30 (0.10)	308	147	16.62
2-Ketoadipate (2KA)	0.94 (0.30)	302	258	17.17
p-OH-phenylpyruvate (PHPP)	0.28 (0.10)	396	179	19.51
Non α-ketoacids				
Glutarate (GA)	0.38 (0.10)	261	158	14.04
p-OH-phenyllactate (PHPL)	0.27 (0.10)	308	293	19.19
Internal standards				
2,2-Dimethylsuccinate (DMS)	0.14 (0.02)	231	275	13.27
2-Ketocaproate (2KC)	0.26 (0.04)	274	200	13.37
Tropate (TA)	0.24 (0.04)	280	267	15.99
Margarate (MGA)	0.07 (0.02)	327	145	21.37
Tetracosane (C24)	0.06 (0.02)	99	127	24.00

2.3. Preparation of urine filter paper and elution of metabolites

The filter paper used was Advantec, UA-5, 50×50 mm. One piece of the filter paper is capable of retaining about 1.2 ml of liquid urine, and samples were blotted to the paper and completely dried at room temperature. At elution of metabolites from the dried filter paper, the folded filter paper was put in a 2.5-ml syringe, and 1.2 ml of distilled water, was spiked to the filter paper. Then the syringe was inserted into a 10-ml test tube, the tube was sealed with parafilm, and the preparation centrifuged at 3000 rpm for 5 min. About 0.8 ml of eluate was obtained. Basically, two pieces of the filter paper were prepared for one analysis. The creatinine concentration of the eluate was determined by Jaffe's method [11], and an aliquot of eluate, including 0.1 mg creatinine, or 1 ml of the eluate from the standard solution, was used for analysis.

2.4. Sample preparation

To 1 ml eluate of the standard solution, or the eluate containing 0.1 mg creatinine, distilled water was added to a total volume of 2 ml, and then 20 μg each of MGA, C24 and/or DMS, 40 μg each of TA and/or 2KC were added as internal standards. Oximation, solvent extraction and TMS derivatization were done, as described [12].

2.5. GC–MS analysis

A capillary GC–MS system, Shimadzu QP 5000 Model (Shimadzu, Kyoto, Japan), equipped with a class 5000 data processing system, and a capillary fused-silica DB-5 column (30 m×0.5 mm I.D.) with a 1.0 μm film thickness of 5% phenylmethyl silicone (J&W Scientific, Folsom, CA, USA) was used. The analytical conditions for GC–MS were as described [12].

2.6. Stability of compounds in dried filter paper

The stability of α-ketoacids and other organic acids in the filter paper was determined on day 0 (the day at preparation), day 7, day 14 and day 28 after preparation with six samples for each. Quantification was expressed as a relative peak area (RPA, %) to

that of internal standard (I.S.) (MGA was usually used).

2.7. Patients' samples

Urine samples from nine children with five types of metabolic disorders were analyzed to determine the practicability of this method.

2.8. Statistics

Student's *t*-test was used to determine the statistical significance.

3. Results

3.1. Comparison of recovery between liquid and filter paper samples

The recovery of α-ketoacids in liquid solution and filter paper eluate was compared six times in inter-assays. Fig. 1 illustrates total ion current (TIC) chromatograms of the standard solution of liquid and filter paper eluate, respectively. As shown in Fig. 2, no significant difference in quantification was seen in six kinds of α-ketoacids, whereas recoveries of PP,

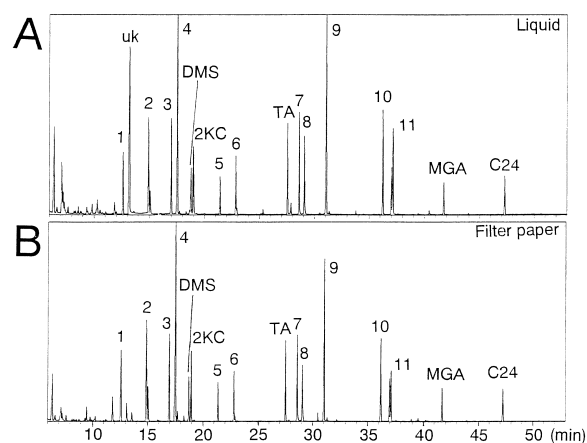Fig. 1. TIC chromatograms of standard solution. Total ion chromatograms of liquid sample (A) and filter paper sample (B). Peaks: 1=pyruvate; 2=2-ketoisovalerate; 3=2-keto-3-methylvalerate; 4=2-ketoisocaproate; 5=glutarate; 6=succinylacetone; 7=2-ketoglutarate; 8=phenylpyruvate; 9=2-ketoadipate; 10=*p*-OH-phenyllactate; 11=*p*-OH-phenylpyruvate. DMS, 2KC, TA, MGA, and C24 were internal standards.
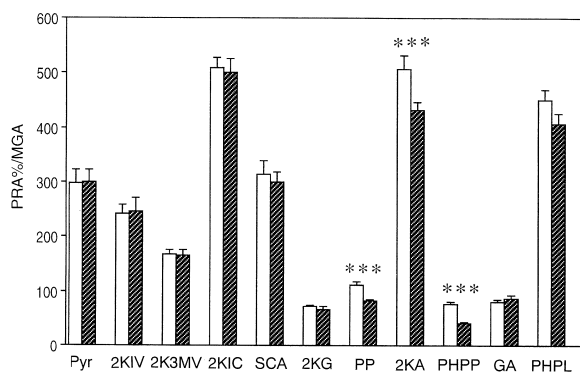
Fig. 2. Comparison of recovery of α-ketoacids between liquid and filter paper. Open and closed columns are liquid and filter paper samples, respectively. Abbreviations as in Fig. 1.
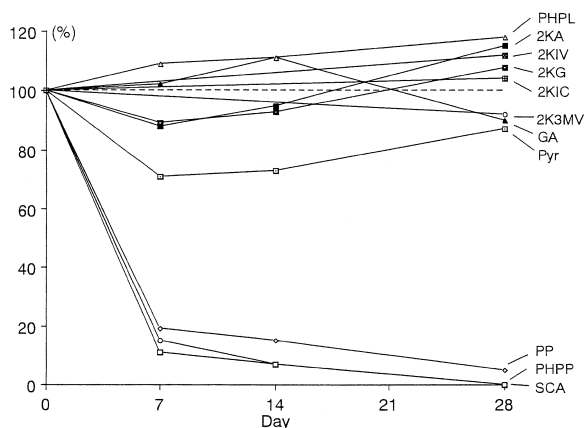

Fig. 3. Stability of compounds in filter paper specimens. The stability was determined up to day 28 and represented as % changes of those on day 0. Abbreviations as in Fig. 1. ***: Significant difference ($P<0.0001$).

2KA and PHPP in the filter paper were significantly decreased compared with those in liquid samples ($P<0.0001$). GA and PHPL, non α-ketoacids, which were added for reference showed no difference in recovery.

3.2. Stability of α-ketoacids in filter paper kept at room temperature

The stability of α-ketoacids in filter paper was determined using the above standard solution and was analyzed on days 0, 7, 14 and 28 after sample preparation. The RPA values (%) on the mass chromatogram at the starting point (day 0) were converted to ''100'' and compared as percentage changes. As shown in Fig. 3, the mean values for

each compound were plotted. The stability of 2KA, 2KIV, 2KG, 2KIC, 2K3MV and Pyr was favorable, and values were at least over 70% of the starting point up to day 28. PP, PHPP and SCA were unstable with percentages of 19, 11 and 15% on day 7; 15, 7 and 7% on day 14; and 5, 0 and 0% on day 28, respectively. PP, as well as phenyllactate (PL), should be elevated in patients with phenylketonuria. PHPP, as well as PHPL, may be increased in ''tyrosinemia'' or ''tyrosyluria''. Increased excretion of PP or PHPP is not essential for PKU or tyrosyluria, if elevation of PL or PHPL is sufficient. Elevation of SCA is essential for the chemical diagnosis of tyrosinemia type 1, so it is likely

Table 2
Comparison of internal standards in quantification of α-ketoacids: relative standard deviation (RSD)

α-Ketoacid	RSD (%) (mean±SD)			
	DMS	2KC	TA	MGA
Pyr	5.9 (233.7±4.6)	5.6 (215.6±4.1)	7.2 (94.3±2.3)	7.6 (300.7±7.6)
2KIV	8.1 (190.5±5.1)	7.8 (175.8±4.6)	9.8 (76.9±2.5)	10.4 (245.3±8.5)
2K3MV	3.8 (128.7±1.6)	5.1 (118.8±2.0)	5.7 (51.9±0.9)	5.9 (165.6±3.3)
2KIC	2.7 (388.4±3.5)	3.8 (358.6±4.5)	4.8 (156.8±2.5)	5.0 (499.7±8.4)
SCA	3.5 (232.8±2.7)	6.0 (215.0±4.3)	6.4 (94.0±2.0)	6.6 (299.8±6.6)
2KG	4.6 (64.9±0.9)	3.5 (59.9±0.7)	5.0 (26.2±0.4)	4.1 (83.4±1.1)
PP	3.6 (63.9±0.8)	5.4 (58.9±1.1)	5.1 (25.8±0.4)	4.0 (82.1±1.1)
2KA	2.8 (336.8±3.1)	3.7 (310.8±3.8)	3.6 (135.9±1.6)	3.4 (432.9±4.9)
PHPP	3.5 (32.3±0.4)	6.5 (29.9±0.7)	4.9 (13.0±0.2)	4.1 (41.6±0.6)

The data were obtained by six times inter-assay. Abbreviations as in Table 1. The unit of ''mean±SD'' is RPA % to various internal standards.

difficult to diagnose tyrosinemia type 1 by this method.

3.3. Comparison of various internal standards for quantification

To determine the appropriate internal standard for quantification of α-ketoacids, internal standards including DMS, 2KC, TA and MGA, were compared. As shown in Table 2, the relative standard deviation (RSD) of the quantified values of α-ketoacids with the four kinds of internal standards was from 2.7 to 8.1% for DMS; from 3.5 to 7.8% for 2KC; from 3.6 to 9.8% for TA; and from 4.0 to 10.4% for MGA, respectively.

3.4. Analysis of urine samples containing significant amounts of α-ketoacids

Urine filter paper samples from one case each of tyrosinemia type 1 and tyrosinemia type 2, two cases each of maple syrup urine disease (MSUD) and

Table 3

Results of diagnostic marker metabolites in subjects from patients with known diseases

	Day 0	Day 14	Day 28
Tyrosinemia type 1			
SCA	1.4	n.d.	–
PHPP	72.9	47.9	–
PHPL	471.3	474.4	–
Tyrosinemia type 2			
PHPP	2.2	n.d.	–
PHPL	845.1	895.5	–
Transient tyrosinemia (case 1)			
PHPP	33.9	4.1	n.d.
PHPL	686.8	781.0	908.9
Transient tyrosinemia (case 2)			
PHPP	20.8	6.9	n.d.
PHPL	448.8	502.1	459.9
Maple syrup urine disease (case 1)			
2-OH-isovalerate	12.2	5.5	–
2K3MV	1.6	0.8	–
2KIC	0.7	n.d.	–
Maple syrup urine disease (case 2)			
2-OH-isovalerate	–	102.6	–
2KIV	–	54.7	–
2-OH-isocaproate	–	5.7	–
2-OH-3-methylvalerate	–	5.6	–
2K3MV	–	140.1	–
2KIC	–	466.2	–
Lactic/pyruvic aciduria (case 1)			
Lactate	73.1	56.1	93.9
Pyruvate	67.5	45.5	39.2
Lactic/pyruvic aciduria (case 2)			
Lactate	285.1	179.8	142.4
Pyruvate	210.4	164.5	178.4
Lactic/pyruvic aciduria (case 3)			
Lactate	191.0	192.5	220.3
Pyruvate	53.3	44.6	35.4

Unit, relative peak area (%) to MGA (internal standard); n.d., not detectable; –, not determined.

transient tyrosinemia and three cases of lactic/pyruvic aciduria were tested on days 0, 14 or day 28. The results are shown in Table 3. For tyrosinemia type 1, succinylacetone was not detectable on day 14. In tyrosinemia type 2 and transient neonatal tyrosinemia, PHPP was reduced on day 14 and was not detectable on day 28. Elevated PHPL is sufficient in identifying "tyrosinemia" or "tyrosyluria", but

ID	Compound	Value	Normal	Range				Factor
1	Lactate-2	59.75	(0.80	0.00	--	4.70	)	74.68
6	2-OH-butyrate-2	7.71	(0.00	0.00	--	0.00	)	?
9	Pyruvate-OX-2	38.16	(4.50	0.00	--	24.10	)	8.48
11	3-OH-butyrate-2	160.91	(0.70	0.00	--	3.70	)	229.87
13	2-OH-isovalerate-2	102.59	(0.00	0.00	--	0.00	)	?
17	2-Keto-isovalerate-OX-2	54.73	(0.00	0.00	--	0.10	)	?
22	2-OH-isocaproate-2	5.70	(0.00	0.00	--	0.00	)	?
25	2-OH-3-methylvalerate-2	5.59	(0.00	0.00	--	0.00	)	?
29	2-Keto-3-methylvalerate-2	140.09	(0.00	0.00	--	0.00	)	?
35	2-Keto-isocaproate-OX-2	466.25	(0.00	0.00	--	0.00	)	?
58	Butyrylglycine-1	1.31	(0.00	0.00	--	0.00	)	?
79	Tiglylglycine-1	1.15	(0.10	0.00	--	0.00	)	11.47
87	2-Ketoglutarate-OX-2	6.68	(26.10	3.00	--	102.90	)	0.26
89	4-OH-phenylacetate-2	9.80	(27.10	8.60	--	73.20	)	0.36
103	Homovanillate-2 (HVA)	29.95	(16.30	5.80	--	24.90	)	1.84
115	Vanilmandelate-3 (VMA)	69.92	(46.60	11.70	--	84.60	)	1.50
118	4-OH-phenyllactate (PHPL)-2	180.18	(1.80	0.00	--	7.00	)	100.10
133	Tetracosane (IS3)	51.40	(0.00	0.00	--	0.00	)	?
134	Tropate (IS2)	236.06	(0.00	0.00	--	0.00	)	?

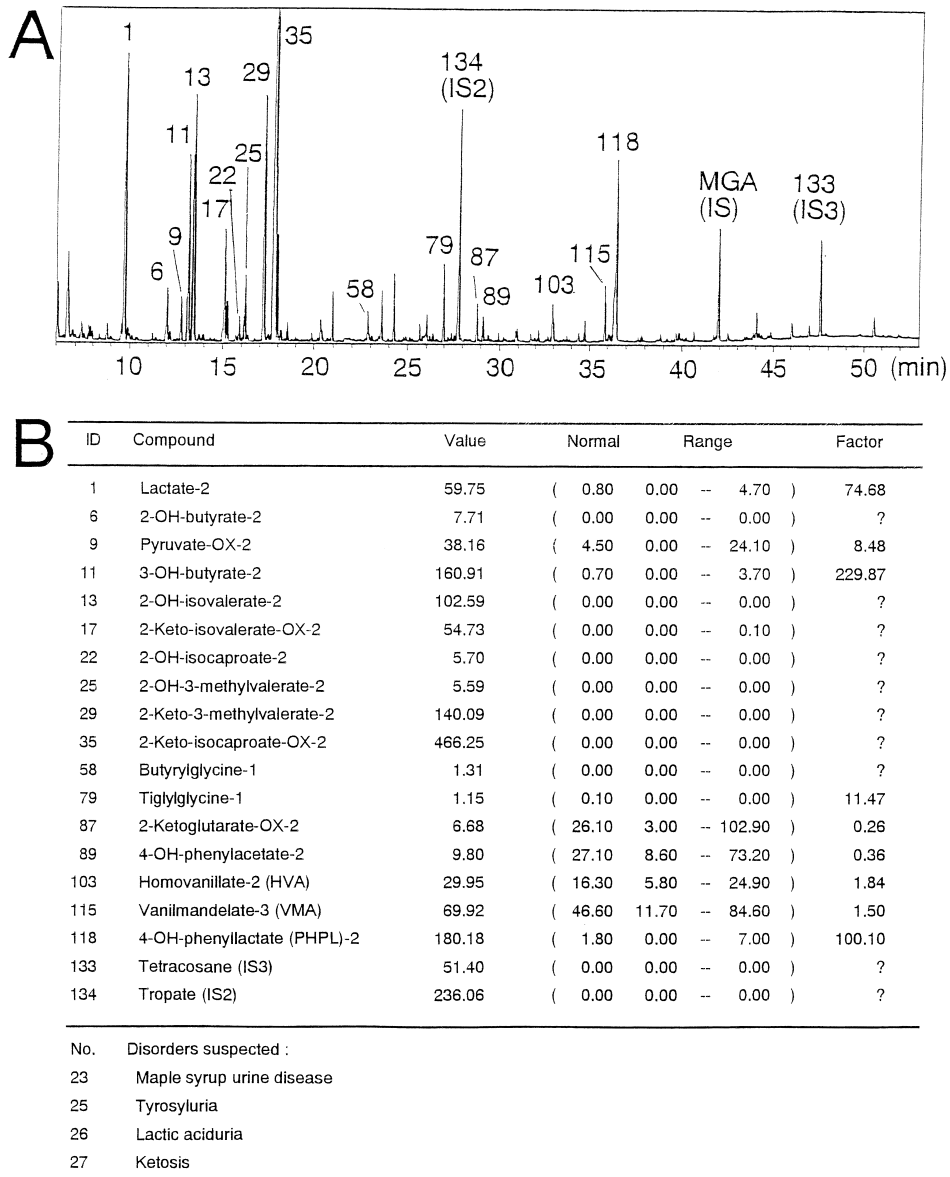| No.	Disorders suspected :
23	Maple syrup urine disease
25	Tyrosyluria
26	Lactic aciduria
27	Ketosis

Fig. 4. Results of analysis of a urine filter paper from a MSUD patient. (A) TIC cromatogram of urinary organic acids; (B) results from the automated data processing system. In the upper and lower parts of Fig. 4B, the results of metabolic profiling and the list of disorders suspected are shown. Abbreviations: ID=peak number matched to that in the chromatogram of (A); Value=relative peak area to that of the internal standard (margarate); Normal and Range=mean value and range in the normal controls, respectively; Factor=value/normal mean value. Names of suspected disorders or pathological conditions, such as MSUD, tyrosyluria, lactic aciduria and ketosis, are indicated at the bottom of (B). Nos. in front of the disease names are the disease numbers that were enrolled in advance.

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insufficient for accurately diagnosing tyrosinemia type 1. The diagnosis of tyrosinemia type 1 requires the elevation of SCA and/or PHPL. MSUD and lactic/pyruvic aciduria could be accurately diagnosed on at least day 14.

A TIC chromatogram and results from our automated GC–MS data processing system of analysis of filter paper urine from an 11-day-old neonate MSUD patient are illustrated in Fig. 4. This was an actual sample sent from India to our laboratory in Japan and we did the analysis 14 days after sample had been taken in India. Lactate, 2-OH-butyrate, Pyr, 3-OH-butyrate, 2-OH-isovalerate, 2KIV, 2-OH-iso-caproate, 2-OH-3-methylvalerate, 2K3MV, 2KIC, butyrylglycine, tiglylglycine and PHPL were the abnormal metabolites detected. Suspected were ''maple syrup urine disease'', ''tyrosyluria'', ''lactic aciduria'' and ''ketosis'' as shown in the bottom of Fig. 4B. Actually, this patient was in an acute stage of MSUD, clinically complicated with lactic acidosis and ketosis, and with neonatal transient tyrosinemia.

4. Discussion

Currently, there are two approaches for the organic acidemia screening, including acylcarnitine analysis by tandem mass spectrometry using dried blood filter paper (tandem MS) [13,14] as well as urinary organic acid analysis by GC–MS [1,2]. Both involve a single assay that can detect multiple disorders. Tandem MS can analyze a larger number of samples per day per instrument, and its running cost are more economical, but the equipment is costly. On the other hand, GC–MS often yields comprehensive and quantitative data, although it requires a longer time for analysis and a higher running cost. Even if the GC–MS is not sufficiently practical to ''mass screening'' for organic acidemias, it will play an important role in diagnosis of high-risk patients, or evaluation of the condition of the patients.

We showed the practicality of urine filter paper with oximation, solvent extraction and trimethyl-silylation, the objective of this study is to develop a simple and convenient screening method for use in clinical organic acidemias. In a comparison of the recovery between liquid and filter paper samples,

three of nine α-ketoacids, PP, PHPP, and SCA, in dried filter paper were unstable when tested on day 7. As mentioned in the Results section, PP, PHPP or SCA are increased in PKU, or tyrosyluria or tyrosinemia type 1. However, elevation of PL is usually sufficient in detection of PKU regardless of the amount of PP; elevation of PHPL indicates ''tyrosinemia'' or ''tyrosyluria'' regardless of PHPP and SCA. The other six α-ketoacids tested were stable for at least 28 days. The data indicated that detection of lactic/pyruvic aciduria, MSUD or α-ketoadipic aciduria is possible with this method. As to internal standards, including DMS, 2KC, TA and MGA, tested for quantification of α-ketoacids, no significant difference was observed. It was suggested that an α-ketoacid such as 2KC for use as an internal standard is not actually needed for α-keto-acid analysis.

Simultaneous analysis of α-ketoacids and non α-ketoacids will be helpful not only for diagnosing disorders, but also more comprehensive evaluation of the metabolic condition of patients, such as secondary lactic/pyruvic aciduria or tyrosyluria. MSUD may be suspected based on the data of amino acid analysis [15]. α-Ketoadpic/α-aminoadipic acidemia may also be based on elevation of α-aminoadipic acid, by amino acid analysis or GC–MS analysis [16]. However, simultaneous analysis of α-ketoacids as well as the other organic acids will allow for a precise pathological diagnosis. The diagnosis of tyrosinemia type 1 would not be precise without findings of increased excretion of SCA, in addition to tyrosyluria.

To date, we identified more than 35 patients with organic acidemias based on samples sent to Japan from China and India using this method. If we are aware of the usefulness and limitation of this method, the GC–MS screening for organic acidemias will be expanded.

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