Journal of Chromatography B, 878 (2010) 942–948

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simplified method for the chemical diagnosis of organic aciduria using GC/MS

Katsuhiro Nakagawa*, Shuichi Kawana, Yuki Hasegawa, Seiji Yamaguchi

Department of Pediatrics, Shimane University School of Medicine, 89-1 En-ya-cho, Izumo, Shimane 693-8501, Japan

ARTICLE INFO

Article history:
Received 3 February 2010
Accepted 12 February 2010
Available online 21 February 2010

Keywords:
Gas chromatograph–mass spectrometer
Organic acidurias
Sample preparation
Solvent extraction
Trimethylsilylation
Flash-heater derivatization

ABSTRACT

GC/MS is widely used for the analysis of urinary organic acids for the chemical diagnosis of organic acidurias such as methylmalonic acidemia, propionic acidemia, isovaleric acidemia, glutaric aciduria type I, and multiple carboxylase deficiency. In this study, a rapid and simple preparation method for this analysis was developed in order to improve the laboratory productivity and the working environment. The solvent extraction and trimethylsilyl derivatization steps of the conventional method were improved by reducing the volume of urine sample and extraction solvent and by applying the flash-heater derivatization, respectively. The new method was successfully applied to the chemical diagnoses of five organic acidurias.

1. Introduction

Due to the expanding newborn screening by electrospray ionization-tandem mass spectrometry, gas chromatograph/mass spectrometry (GC/MS) will be extensively employed for the chemical diagnosis of organic acidurias [1]. For infants with the positive screening result and high-risk infants with a family history of those diseases, the chemical diagnostic method by GC/MS is indispensable [2–4], because a simultaneous analysis of urinary organic acids using GC/MS provides a wide range of metabolite profiling, which is very important for the diagnosis.

However, the major disadvantage of the GC/MS method is that the sample preparation is the most labor intensive and time consuming work of this analysis [5,6]. For the sample preparation, solvent extraction of organic acids in the urine, followed by trimethylsilyl (TMS) derivatization is commonly used [2,7]. There are several disadvantages in such an approach, for example, the time required for the extraction step, and the evaporation of the relatively large volume of extraction solvent, and for the subsequent derivatization reaction, the constant attention that the operators must pay to in performing these procedures, and the exposure of the operators to toxic reagents such as the extraction solvent and the derivatization reagent.

We developed a rapid and simple sample preparation method by improving the conventional solvent extraction and trimethylsilyl derivatization procedures [7]. The volume of extraction solvent and the number of extraction cycles were reduced for the solvent extraction procedure, and a flash-heater derivatization technique [8–15] was used for the TMS derivatization procedure, by injecting derivatization reagent together with the extract into the GC/MS.

The developed method was applied to urine samples obtained from patients with organic acidurias to demonstrate the effectiveness of this method.

2. Experimental

2.1. Chemicals

Since the target compounds possess different properties, certain representative compounds were chosen from commercially obtainable compounds to validate this method, namely: lactic acid, 3-hydroxy-propionic acid, 2-hydroxy-isovaleric acid, 3-hydroxy-isovaleric acid as hydroxyl fatty acid; 3-hydroxy-butyric acid as ketone body; methylmalonic acid, ethylmalonic acid, glutaric acid, adipic acid, sebacic acid and suberic acid as dicarboxylic acid; and 4-hydroxy-phenyllactic acid as aromatic acid. In addition to these, isovalerylglycine and phenyllactic acid were also chosen. All the compounds except 2-hydroxy-isovaleric acid (Japan Sigma–Aldrich Chemical Co., Tokyo, Japan) and isovalerylglycine (Tokyo Chemical Industry Co., Ltd., Osaka, Japan) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Margaric acid (MGA) and tetracosane (C_{24}) for internal standard, urease for decomposition of urea, ethyl acetate for solvent extraction, hydrochloric acid for adjusting pH of urine and N,O-bis(trimethylsiliyl)trifluoroacetamide + 1% trimethyl-chlorosilane

* Corresponding author. Tel.: +81 75 823 1410; fax: +81 75 841 9326.
E-mail addresses: nk@shimadzu.co.jp (K. Nakagawa), s_kawana@shimadzu.co.jp (S. Kawana), yukirin@med.shimane-u.ac.jp (Y. Hasegawa), seijiyam@med.shimane-u.ac.jp (S. Yamaguchi).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.02.020

(BSTFA + 1%TMCS) for TMS derivatization were obtained from Wako Pure Chemical Industries Ltd. The solution of *n*-alkanes (C_9–C_{33}) was obtained from Restek Corp. (Bellefonte, PA, USA).

Internal standard solution of margaric acid (MGA) and tetracosane (C_{24}) were prepared in ethyl acetate (0.5 mg/ml).

2.2. Sample preparation

The method reported by Kimura et al. [7,16] was used as the conventional method. The modified solvent extraction and TMS derivatization were described in Section 2.2.1 and Section 2.2.2, respectively.

2.2.1. Modified solvent extraction

The following preparation was performed in a 1.5 ml-glass vial (Shimadzu GLC Ltd., Tokyo, Japan). An aliquot of urine equivalent to 0.02 mg of creatinine (volume of urine is generally between 0.01 and 0.20 ml) was diluted with distilled water to adjust the final volume to 0.20 ml. To the diluted urine was then added 2 units of urease, and the mixture was incubated for 30 min at 37 °C. Four microliters of the internal standard solution (0.5 mg/ml) was then added. The mixture was acidified with 35 μl of HCl (6N) and shaken for approximately 30 s with a vortex mixer. The organic acids were then extracted with 1.2 ml of ethyl acetate by mixing vigorously for approximately 30 s with a vortex mixer. The mixture was centrifuged at 2010 × *g* for 5 min and the organic layer was treated with anhydrous sodium sulfate (0.5 g) prior to GC/MS analysis.

2.2.2. Flash-heater derivatization

The Flash-heater derivatization used in the new method was carried out as follows. A 1 μl aliquot of the extract (ethyl acetate) was sandwiched between two plugs of the BSTFA + 1%TMCS in a 10 μl injection syringe by drawing in sequence: 1 μl of BSTFA + 1%TMCS, 0.5 μl of air, 1 μl of the extract, 0.5 μl of air and 0.5 μl of BSTFA + 1%TMCS. The sample was then injected into the split/splitless injection port heated at 280 °C.

2.3. Gas chromatograph–mass spectrometer analysis

2.3.1. Gas chromatograph–mass spectrometer

The analysis were performed on a gas chromatograph coupled to a quadrupole mass spectrometer (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan) equipped with an automatic injection system (AOC-20i+s), a split/splitless injection port with a glass liner packed with glass wool (Restek) and a DB-5 capillary column (30 m × 0.25 mm i.d., 1.00 μm, J & W Scientific, Folsom, CA, USA). The analytical conditions of both methods are shown in Table 1.

2.3.2. Data processing

The data was processed using GCMSsolution software and GC/MS metabolite mass spectral database, which includes mass spectra and retention index data; the software and database are from Shimadzu. The retention times of target compounds were calculated from measured retention times of *n*-alkanes (C_{10}–C_{26}) and the retention indices of targets compounds stored in the database. The target compounds in the chromatogram were identified by the calculated retention times and their mass spectra [7,16,17].

2.4. Urine sample analysis

2.4.1. Analysis of urine sample spiked with organic acids

A normal urine sample was spiked with 14 organic acids (Table 2) at 50 μg/ml and internal standard solution at 10 μg/ml, and analyzed by both conventional and new methods. The retention indices of the TMS derivatives of the organic acids were determined using the measured retention times of *n*-alkanes [18].

Table 1
Analytical conditions of the conventional and new methods.

	Conventional GC/MS method	New GC/MS method
Extraction	Conventional liquid extraction	Modified liquid extraction
Derivatization	Oximation, TMS	TMS
Column	DB-5 30 m × 0.25 mm i.d. df = 1.00 μm	DB-5 30 m × 0.25 mm i.d. df = 1.00 μm
Injection method	Conventional injection (1 μl)	Flash injection (1 μl BSTFA + 1%TMCS, 1 μl sample, 0.5 μl BSTFA + 1%TMCS)
Injection temp.	280 °C	280 °C
Split mode	Split mode 1:10	Splitless mode (1 min)
Carrier gas	39 cm/s (constant velocity)	39 cm/s (constant velocity)
Column temp.	100 °C (4 min)–4 °C/min–280 °C (11 min)	100 °C (4 min)–4 °C/min–280 °C (11 min)
Interface temp.	280 °C	280 °C
Ion source	200 °C	200 °C
Data acquisition rate	0.3 s	0.3 s
Data acquisition mode	Scan	SIM

2.4.2. Analysis of normal control sample and determination of cut-off values

Urine samples were collected from 40 normal controls and analyzed by the new method. TMS derivatives of methylmalonic acid, propionylglycine, glutaric acid, isovaleryglycine, 3-methylcrotonylglycine, 3-hydroxyglutaric acid, 2-hydroxyglutaric acid, methylcitric acid were quantified as the diagnostic markers of methylmalonic acidemia (MMA), propionic acidemia (PPA), isovaleric acidemia (IVA), glutaric aciduria type I (GAI) and multiple carboxylase deficiency (MCD). The quantitative results were expressed in the form of the peak area ratios of the target compounds to the internal standard (MGA), and the mean values and the standard deviations were calculated. The cut-off value was defined as the mean plus three standard deviations.

2.5. Chemical diagnosis using the new method

Urine samples were obtained from 20 patients with the following disorders (diagnosed by the conventional method and clinical symptoms): MMA, PPA, IVA, GAI and MCD. To validate the new method each patient sample was analyzed five times and then the solvent of BSTFA + 1%TMCS as the blank sample was analyzed three times to confirm the carry over levels of target compounds.

The derivatives of the diagnostic markers with abnormal values were determined by comparing the values to the cut-off values, and their combinations allowed the chemical diagnosis [7,16,17].

3. Results

3.1. Analysis of urine sample spiked with organic acids

Table 2 shows the retention indices and the peak area ratio of the trimethylsilyl (TMS) derivatives to C_{24} (which is not derivatized) for all the TMS derivatives which were detected after five analyses of a urine sample spiked with the organic acids using the conventional and new methods.

Derivatives with different numbers of TMS-groups were formed and the main product differed between the two methods. By using the conventional method, 18 TMS derivatives were detected

Table 2
Within-run repeatability and ratio of relative recovery for organic acids added to urine.

No.	Compound[a]	*m/z*		Conventional method (n = 5)				New method (n = 5)				Ratio[b]
		Q-ion[c]	C-ion[c]	R.I.[d]	Peak area ratio			R.I.[d]	Peak area ratio			
					Mean[e]	SD[e]	%RSD[e]		Mean[f]	SD[f]	%RSD[f]	
1	3-Hydroxyisovaleric acid-TMS*	117	175	1032	2.440	0.570	23.4	1036	35.445	2.640	7.4	14.53
2	3-Hydroxyisovaleric acid-diTMS*	247	205	1213	4.359	0.243	5.6	1214	0.136	0.017	12.2	0.03
3	2-Hydroxyisovaleric acid-TMS*	175	157	1047	0.005	0.006	112.9	1051	0.179	0.031	17.4	35.88
4	2-Hydroxyisovaleric acid-diTMS*	219	247	1169	5.270	0.199	3.8	1171	4.605	0.268	5.8	0.87
5	Lactic acid-diTMS	219	191	1060	1.140	0.124	10.8	1062	2.991	0.324	10.8	2.62
6	3-Hydroxypropionic acid-diTMS	177	219	1144	3.731	0.236	6.3	1146	3.390	0.212	6.3	0.91
7	3-Hydroxybutyric acid-diTMS	191	233	1161	4.567	0.232	5.1	1163	1.503	0.126	8.4	0.33
8	Methylmalonic acid-diTMS	247	218	1218	4.210	0.158	3.8	1218	3.015	0.286	9.5	0.72
9	Ethylmalonic acid-diTMS	261	217	1284	5.182	0.155	3.0	1284	3.555	0.295	8.3	0.69
10	Phenyllactic acid-TMS*	205	194	1399	0.008	0.004	58.7	1399	0.029	0.045	153.4	3.83
11	Phenyllactic acid-diTMS*	194	267	1517	10.123	0.262	2.6	1517	6.966	0.381	5.5	0.69
12	Glutaric acid-diTMS	261	158	1405	11.372	0.315	2.8	1404	7.471	0.384	5.1	0.66
13	Isovalerylglycine-TMS*	216	189	1488	1.075	0.075	7.0	1487	2.965	0.185	6.2	2.76
14	Isovalerylglycine-diTMS*	288	176	1519	7.936	0.118	1.5	–	0.000	0.000	–	0.00
15	Adipic acid-diTMS	275	111	1508	6.609	0.177	2.7	1507	3.592	0.169	4.7	0.54
16	Suberic acid-diTMS	303	187	1702	7.573	0.193	2.5	1700	3.552	0.203	5.7	0.47
17	Sebacic acid-diTMS	331	215	1897	17.164	0.346	2.0	1896	6.926	0.278	4.0	0.40
18	4-Hydroxyphenyllactic acid-triTMS	308	293	1918	26.045	0.465	1.8	1917	7.023	0.313	4.5	0.27
19	Margaric acid-TMS	327	145	2145	1.918	0.036	1.9	2146	1.356	0.102	7.5	0.71
20	Tetracosane	99	67	2400	1.000	0.000	0.0	2400	1.000	0.000	0.0	1.00

[a] An asterisk indicates that two TMS derivatives were formed form an organic acid.
[b] Ratio means the relative recovery and based on ratio of the peak area ratio in the new and conventional methods.
[c] Q- and C-ions were used for selected ion monitoring (SIM).
[d] R.I. = retention index.
[e] Mean, SD and %RSD were calculated based on the peak area ratio (TMS derivatives to C24) after five analyses by the conventional method.
[f] Mean, SD and %RSD were calculated based on the peak area ratio (TMS derivatives to C24) after five analyses by the new method.

from 14 organic acids: two TMS derivatives were obtained from 3-hydroxyisovaleric acid (-TMS, -diTMS), 2-hydroxyisovaleric acid (-TMS, -diTMS), phenyllactic acid (-TMS, -diTMS) and isovaleryglycine (-TMS, -diTMS). The %RSD of the peak area ratios (TMS derivatives to C_{24}) ranged from 1.5 to 10.8% except 3-hydroxyisovaleric acid-TMS (23.4%), 2-hydroxyisovaleric acid-TMS (112.9%), phenyllactic acid-TMS (58.7%). On the other hand, by the new method 17 TMS derivatives were detected from 14 organic acids: two TMS derivatives were obtained from 3-hydroxyisovaleric acid (-TMS, -diTMS), 2-hydroxyisovaleric acid (-TMS, -diTMS) and phenyllactic acid (-TMS, -diTMS). The within-run repeatability (%RSD of the peak area ratios) ranged from 4.0 to 12.2% except 2-hydroxyisovaleric acid-TMS (17.4%) and phenyllactic acid-TMS (153.4%). Additionally the analysis was carried out every 2 days over a 10 days period and the inter-run reproducibility (%RSD of the peak area ratios) ranged from 3.4 to 13.1% for the main products.

The differences in retention indices between the two methods ranged from −2 to 4 for the same TMS derivatives.

In order to compare the recovery of the added organic acids between the two methods, the peak area ratios (organic acid to C_{24}) obtained from the new method was divided by those from the conventional method (Table 2). Those values showed more than 1.2 for 5 compounds, 0.80–1.20 for 2 compounds and less than 0.80 for 11 compounds. Additionally the recovery in percentage of the spiked value ranged from 24.6% to 115.0%.

3.2. Analysis of normal control sample and determination of cut-off values

Table 3 shows the quantitative results of the normal urine analysis and the cut-off values which were determined by the mean plus three standard deviations of the relative peak area (target compound to MGA). If the target compounds were not detected in the urine sample, the cut-off value was treated as 0.5% (the quantitative limit of MGA).

3.3. Chemical diagnosis using the new method

Table 4 shows the quantitative results for urine samples of individual patients with MMA, PPA, GAI, IVA and MCD obtained by the new method, each analyzed in five replicates. Twelve target compounds (TMS derivatives of the diagnostic markers) were detected and the %RSD ranged from 4.3% to 12.0%. Propionylglycine-diTMS, isovalerylglycine-diTMS and 3-methylcrotonylglycine-diTMS (for PPA, IVA and MCD, respectively) were not detected. However, propionylglycine-TMS, isovalerylglycine-TMS, and 3-methylcrotonylglycine-TMS were detected, and the %RSDs were 4.3%, 5.1% and 11.6%, respectively.

Carry over of diagnostic markers were not observed either as memory effects or ghosting.

As an example, results for a patient with MCD are summarized in Fig. 1 and Table 5. Fig. 1 shows the total ion chromatogram, and Table 5 provides the quantitative results and the comparison to the cut-off value. For all evaluated patient samples the chemical diagnostic results are summarized in Table 6. These results were similar to those of the chemical diagnosis by the conventional method and the clinical symptom.

4. Discussion

In this study, we simplified the solvent extraction and the TMS derivatization of the conventional method [7] by considering the advantages including the appropriate sample clean-up and the established data interpretation [7,16,17]. For sample preparations, aside from liquid extraction and direct/urease [19,20] methods have been developed. Among these, the urease method developed by Matsumoto and Kuhara [20] is the simplest and most efficient in reducing the volume of urine sample and organic solvent. This includes urease treatment to remove urea, deproteinization with ethanol, evaporation to dryness and trimethylsilylation. Another distinctive feature of this method is that both amino acids and sugars can be extracted from urine together with organic acids and

Table 3
Cut-off value obtained from forty normal controls.[a].

No.	Compound[b]	R.I.[c]	*m/z*		Peak area ratio (%)			
			Q-ion[d]	C-ion[d]	Mean[e]	SD[e]	Range[e]	Cut-off[f]
1	Methylmalonic acid-diTMS	1219	247	218	3.48	2.00	0.00–9.93	9.49
2	Propionylglycine-diTMS*	1359	188	159	0.63	0.87	0.00–3.36	3.23
3	Propionylglycine-diTMS*	1428	260	232	0.00	0.00	0.00–0.00	0.50
4	Glutaric acid-diTMS	1404	261	158	6.39	3.78	1.85–15.85	17.74
5	Isovalerylglycine-TMS*	1488	216	189	1.03	1.67	0.00–6.73	6.03
6	Isovalerylglycine-diTMS*	1520	288	176	0.00	0.00	0.00–0.00	0.50
7	3-Methylcrotonylglycine-TMS*	1564	214	229	0.00	0.00	0.00–0.00	0.50
8	3-Methylcrotonylglycine-diTMS	1578	286	184	0.00	0.00	0.00–0.00	0.50
9	3-Hydroxyglutaric acid-triTMS	1582	247	349	0.00	0.00	0.00–0.00	0.50
10	2-Hydroxyglutaric acid-triTMS	1583	349	203	1.20	0.64	0.00–2.79	3.13
11	Methylcitric acid-tetraTMS*	1862	389	479	0.00	0.00	0.00–0.00	0.50
12	Methylcitric acid-tetraTMS*	1874	389	479	0.00	0.00	0.00–0.00	0.50
13	Margaric acid-TMS	2146	327	145	100.00	0.00	100.00–100.00	100.00
14	Tetracosane	2400	99	67	71.53	13.15	55.75–125.60	110.97

[a] Healthy children less than 3 months.
[b] An asterisk indicates that two TMS derivatives were formed from an organic acid.
[c] R.I. = retention index.
[d] Q- and C-ions were used for selected ion monitoring (SIM).
[e] Mean, SD and range were calculated based on the peak area ratio (TMS derivatives to MGA, %) for 40 normal controls.
[f] Cut-off value was defined as the mean plus three standard deviations of peak area ratio (target compound to MGA).

used for chemical diagnosis. However, large amounts of impurities are extracted simultaneously, thus complicating the organic acid peak identification. On the other hand, the solvent extraction method enables a rapid and accurate identification and prevents column and ion source contamination by removing impurities including sulfur and phosphoric acid originated from urine. This is one of the reasons why the solvent extraction method has been widely used in clinical laboratories [5,6,21]. Therefore, we simplified the solvent extraction procedure used in the conventional method.

To simplify the solvent extraction, reduction of the volume of urine sample and the number of extractions performed is the most effective if the organic acids are to be extracted quantitatively and detected. In the new method, the volume was reduced from 2 ml to 0.2 ml and the volume of extraction solvent (ethyl acetate) was proportionally reduced from 12 ml to 1.2 ml. The number of extractions performed was reduced from two to one.

Moreover, the TMS derivatives of diagnostic markers for the evaluated aciduria could be detected without oximation process in the conventional method. However, this method cannot be applied to the chemical diagnosis in which keto acids are indispensable as the diagnostic markers, for example, succinylacetone for tyrosinemia type I or branched-chain alpha-keto acids for maple syrup urine disease.

To simplify the TMS derivatization, the flash-heater derivatization was applied; this technique is widely used for methylation of drugs [9], TMS derivatization of morphine [10] and TMS derivatization of fatty acid [11,22,23]. In the new method, a 1 μl aliquot of the extract (ethyl acetate) was sandwiched between two plugs of the BSTFA + 1%TMCS in the injection syringe and injected into the glass liner with glass wool at 280 °C as mentioned in Section 2.2.2 (Flash-heater derivatization). The organic acids were derivatized in the glass liner and the front part of the capillary column immediately before gas chromatographic analysis. On the other hand, in the conventional method, the extraction solvent of approximately 12 ml is evaporated under a stream of nitrogen gas and the residue was trimethylsilylated with 0.1 ml of BSTFA + 1%TMCS for 30 min at 80 °C. As the results, the evaporation step after the extraction could be omitted and the volume of BSTFA + 1%TMCS could be greatly reduced for each analysis (approximately 1/70 reduction) by using

Table 4
Quantitative results of TMS derivatives of diagnostic markers in urine sample obtained from patients.

Sample[a]	Compound[b]	Peak area ratio (%)[c]		
		Mean	SD	%RSD
MMA	Methylmalonic acid-diTMS	1456.1	116.4	8.0
PPA	Propionylglycine-TMS*	47.2	2.0	4.3
	Propionylglycine-diTMS*	0.0	0.0	–
	2-Hydroxyglutaric acid-triTMS	16.7	0.6	3.4
	Methylcitric acid-tetraTMS* (1)[d]	6.6	0.5	7.0
	Methylcitric acid-tetraTMS* (2)[d]	4.1	0.4	9.6
GAI	Glutaric acid-diTMS	4568.4	369.3	8.1
	3-Hydroxyglutaric acid-triTMS	21.3	2.5	12.0
IVA	Isovalerylglycine-TMS*	1010.6	51.0	5.1
	Isovalerylglycine-diTMS*	0.0	0.0	–
MCD	3-Methylcrotonylglycine-TMS*	6.3	0.7	11.6
	3-Methylcrotonylglycine-diTMS*	0.0	0.0	–

[a] Sample was obtained from patients diagnosed by the conventional method and clinical symptom. *Abbreviations of diseases*: MMA, methylmalonic acidemia; PPA, propionic acidemia; GAI, glutaric aciduria type I; IVA, isovaleric acidemia; MCD, multiple carboxylase deficiency.
[b] An asterisk indicates that more than two TMS derivatives were formed form an organic acid.
[c] Quantitative results are expressed in the form of the peak area ratio (TMS derivatives to MGA, %). Mean, SD and %RSD were calculated by five analyses.
[d] Methylcitric acid-tetraTMS (1) and (2) are isomers.

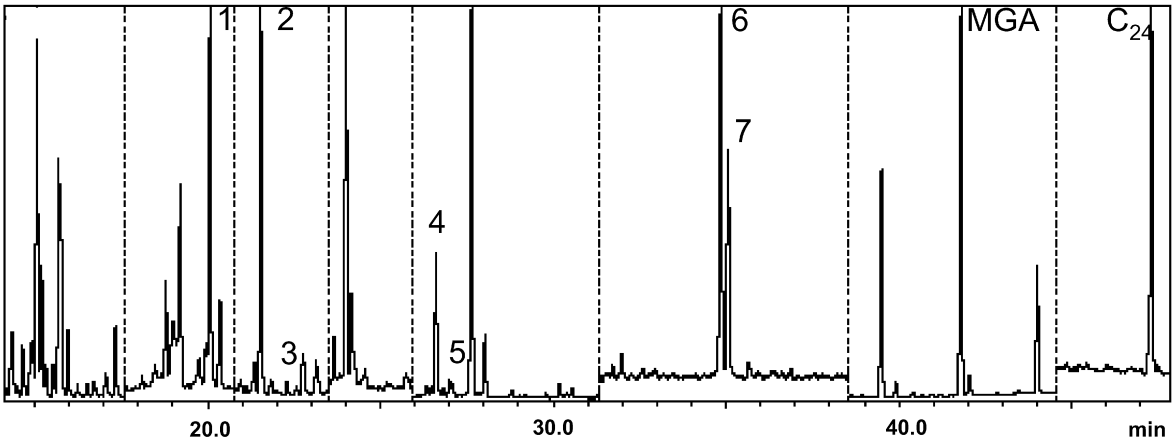

Fig. 1. Total ion chromatogram of SIM of urine sample obtained from a patient with multiple carboxylase deficiency using the new method. Using the new method, urine sample obtained from a patient with multiple carboxylase deficiency was analyzed. The following target compounds were detected: (1) propionylglycine-TMS; (2) glutaric acid-diTMS; (3) propionylglycine-diTMS; (4) 3-methylcrotonylglycine-TMS; (5) 3-methylcrotonylglycine-diTMS; (6) methylcitric acid-tetraTMS; (7) methylcitric acid-tetraTMS; MGA = margaric acid-TMS (I.S.); C_{24} = tetracosane (I.S.). I.S.: internal standard.

the new method. The chance of exposing the operator to extraction solvent and derivatization reagent could also be minimized.

The evaluated organic acids added to urine sample, which formed only one derivative, showed excellent repeatability (%RSD less than 10.8%) (Table 2). These results indicate that those organic acids were extracted quantitatively and completely derivatized by the new method. However, the minimum ratio of the relative recoveries (the new to conventional methods) was 0.27 (4-hydroxyphenyllactic acid-triTMS). In order to cope with the lower recovery, selected ion monitoring mode (SIM) was used for the mass spectrometer measurement.

3-Hydroxyisovaleric acid, 2-hydroxyisovaleric acid, phenyllactic acid and isovalerylglycine formed two TMS derivatives in both methods (Table 2). With the new method, the main products such as 3-hydroxyisovaleric acid-TMS and isovalerylglycine-TMS tended to form derivatives with fewer TMS-groups in contrast to the conventional method, which produced derivatives with more TMS-groups as the main product such as 3-hydroxyisovaleric acid-diTMS and isovalerylglycine-diTMS. In spite of the number of TMS-groups, the repeatabilities of the main products were less than 7.4% in the new method and the ratios of relative recoveries were much higher than those of the by-products. Additionally, the carryover of diagnostic markers was not detected for the patients' sample which contained diagnostic markers at high concentrations. These results indicate that those organic acids were also extracted and derivatized quantitatively.

In the patients' urine analysis (Table 4), the repeatabilities of the main TMS derivatives of the diagnostic markers were excellent (%RSD not over 12%) similar to the analysis of urine sample spiked with representative organic acids (Table 2).

From these results, it can be concluded that the new method could be applied to the chemical diagnosis.

As described above, when multiple TMS derivatives were formed, the main TMS derivative could be chosen as the TMS derivatives of the diagnostic marker (the target compound) similar to the conventional method. Propionylglycine-diTMS (related to PPA), isovalerylglycine-diTMS (to IVA) 3-methylcrotonylglycine-diTMS (to MCD), which are the TMS derivatives of the diagnostic markers

Table 5
Quantitative results[a] of urine obtained from a patient with multiple carboxylase deficiency.

No.	Compound[b]	Patient	Control[c] (%) (n = 40)			
			Mean	Range	Cut-off	Factor[d]
1	Methylmalonic acid-diTMS	8.10	3.48	0.00–9.93	9.49	0.85
2	Propionylglycine-TMS*	27.32	0.63	0.00–3.36	3.23	8.45*
3	Propionylglycine-diTMS*	0.69	0.00	0.00–0.00	0.50	1.38*
4	Glutaric acid-diTMS	42.95	6.39	1.85–15.85	17.74	2.42*
5	Isovalerylglycine-TMS*	0.00	1.03	0.00–6.73	6.03	0.00
6	Isovalerylglycine-diTMS*	0.00	0.14	0.00–5.75	2.87	0.00
7	3-Methylcrotonylglycine-TMS*	30.30	0.00	0.00–0.00	0.50	60.60*
8	3-Methylcrotonylglycine-diTMS*	5.63	0.00	0.00–0.00	0.50	11.27*
9	3-Hydroxyglutaric acid-triTMS	0.00	0.00	0.00–0.00	0.50	0.00
10	2-Hydroxyglutaric acid-triTMS	1.33	1.20	0.00–2.79	3.13	0.42
11	Methylcitric acid-tetraTMS* (1)[e]	2.92	0.00	0.00–0.00	0.50	5.84*
12	Methylcitric acid-tetraTMS* (2)[e]	1.91	0.00	0.00–0.00	0.50	3.83*
13	Margaric acid-TMS	100.00	100.00	100.00–100.00	100.00	1.00
14	Tetracosane	74.99	71.53	55.75–125.60	110.97	0.68
Interpretation: Multiple carboxylase deficiency						

[a] Quantitative results are expressed in the form of the peak area ratio (TMS derivatives to MGA, %).
[b] An asterisk indicates that two TMS derivatives were formed form an organic acid.
[c] Mean, range and cut-off values based on the peak area ratio (TMS derivatives to MGA) were obtained from 40 normal controls (Table 3).
[d] Factor is the ratio of the measured to the cut-off values. If the measured value was more than the cut-off value, the compounds judged as abnormal and marked with an asterisk.
[e] Methylcitric acid-tetraTMS (1) and (2) are isomers.

Table 6
Chemical diagnostic results by the new method.

Patient[a]	Diagnostic result		Compound name[b]	Mark[c]	Factor range[d]
	Conventional[e]	New[f]			
MMA	4	4	Methylmalonic acid-diTMS	4	3.52–326.58
PPA	4	4	Propionylglycine-TMS	1	0.00–15.28
			Methylcitric acid-tetraTMS (1)[g]	4	2.71–25.61
			Methylcitric acid-tetraTMS (2)[g]	4	3.15–15.33
GAI	5	5	Glutaric acid-diTMS	5	1.42–654.15
			3-Hydroxyglutaric acid-triTMS	5	17.60–58.60
IVA	3	3	Isovalerylglycine-TMS	3	4.45–297.32
MCD	4	4	3-Methylcrotonylglycine-TMS	4	60.60–158.56
			3-Methylcrotonylglycine-diTMS	4	11.27–42.01
			Methylcitric acid-tetraTMS (1)[g]	3	1.08–5.84
			Methylcitric acid-tetraTMS (2)[g]	4	1.13–4.29

[a] *Abbreviations of diseases*: MMA, methylmalonic acidemia; PPA, propionic acidemia; GAI, glutaric aciduria type I; IVA, isovaleric acidemia; MCD, multiple carboxylase deficiency.
[b] TMS derivatives used as the target compounds for the chemical diagnosis.
[c] The number of samples which gave a higher quantitative result than the cut-off value for each target compound.
[d] Factor range is the range of ratio (the measured to the cut-off values) for the target compound. If the ratio was more than one, the compounds judged as abnormal.
[e] The number of patients diagnosed by the conventional method and clinical symptom.
[f] The number of patients diagnosed by the new method. Both the conventional and new methods showed the same diagnostic results for all patients.
[g] Methylcitric acid-tetraTMS (1) and (2) are isomers.

by the conventional method, were not detected by the new method (Table 4). However, propionylglycine-TMS, isovalerylglycine-TMS and 3-methylcrotonylglycine-TMS were detected as the corresponding TMS derivatives and the repeatabilities (%RSDs) obtained by the new method were less than 12.0%. The main products of acylglycines in the new method were TMS derivatives with fewer TMS-groups because the reaction time in the liner was shorter than that in the conventional method. Based on these results, it can be concluded that these TMS derivatives could be used for chemical diagnosis.

As the TMS derivatives and their relative recoveries obtained by using the new method were not the same as those obtained by using the conventional method (Table 2), the cut-off value for the conventional method could not be applied to the new method. Therefore, the cut-off values should be determined from the analysis of normal controls (Table 3).

Both the new method and the estimated cut-off values (Table 3) were successfully applied to the chemical diagnoses of 20 patients. For example, MCD is a disorder of biotin metabolism, resulting in impaired activities of the four biotin-dependent carboxylases: propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase and acetyl-CoA carboxylase. The urinary organic acid analysis of the patient revealed an elevation of 3-methylcrotonylglycine and methylcitric acid, and an absence of methylmalonic acid [7]. The results from the quantitative analysis of urine obtained from a patient with MCD showed that 3-methylcrotonylglycine and methylcitric acid were detected as higher than the cut-off values and methylmalonic acid was detected as lower than the cut-off values (Table 5). Although 3-methylcrotonylglycine-diTMS was not detected in another patient's urine in Table 4, it was detected in this patient's urine owing to the higher concentration. Therefore, by using the new method, we could diagnose that the patient had MCD, which was also the chemical diagnosis obtained from the conventional method and the clinical symptoms. For the other patients with the five different diseases, the chemical diagnosis results agreed with those of the conventional method and their clinical symptoms (Table 6). Although the %RSDs in the new method are larger than those in the conventional method in evaluated samples, these results showed that the new method can be applied to the chemical diagnosis of methylmalonic acidemia, propionic acidemia, isovaleric acidemia, glutaric aciduria type I and multiple carboxylase deficiency. Further studies may be needed before the new method can be applied to routine analysis, especially for the cases where lower analytical errors are required [24], such as for therapy monitoring and for the diagnosis of patients as having moderate hyper-excretions or not in an acute episode.

Since the organic acids were not concentrated in the new method, SIM was used for mass spectrometry to improve sensitivity [24]. All urine samples from patients could be analyzed without missing the target compounds. However, the concentrations of target compounds excreted to urine depended on the patient's condition and the enzyme activity. In order to eliminate any undetectable target compounds at trace levels of concentration and prevent the carryover of higher concentration levels, the concentration range must be confirmed by analyzing more real samples.

In future, we will expand this method to other organic aciduria by confirming recovery and repeatability and estimating the cut-off value.

5. Conclusion

This new method enables simple, rapid and safe sample preparation for urinary organic acid analysis using GC/MS by reducing the total volume of organic solvent and derivatization reagents and by the flash-heater derivatization technique. It was successfully applied to 20 patients with the 5 organic acidurias, providing the same chemical diagnosis results as the conventional method. This method will be useful for the chemical diagnosis of organic acidurias and also the clinical applications of urinary organic analysis because of easy and safe sample preparations.

References

[1] Y. Hasegawa, M. Iga, M. Kimura, Y. Shigematsu, S. Yamaguchi, J. Chromatogr. B 823 (2005) 13.
[2] S.I. Goodman, S.P. Markey, Diagnosis of Organic Acidemias by Gas Chromatograpy–Mass Spectrometry, Alan R. Liss, Inc., New York, 1981.
[3] R.A. Chalmers, A.M. Lawson, Organic Acids in Man, Chapman and Hall, London, 1982.
[4] T. Kuhara, J. Chromatogr. B 758 (2001) 3.
[5] K. Tanaka, A. West-Dull, D.G. Hine, T.B. Lynn, T. Lowe, Clin. Chem. 26 (1980) 1847.
[6] Y. Mardens, A. Kumps, C. Planchon, C. Wurth, J. Chromatogr. 577 (1992) 341.
[7] M. Kimura, T. Yamamoto, S. Yamaguchi, Tohoku J. Exp. Med. 188 (1999) 317.
[8] J.M. Halket, V.G. Zaikin, Eur. J. Mass Spectrom. 9 (2003) 1.
[9] S. Ahuja, J. Pharm. Sci. 65 (1976) 163.

[10] K.E. Rasmussen, J. Chromatogr. 120 (1976) 491.
[11] A.S. Christophersen, K.E. Rasmussen, F. Tønnesen, J. Chromatogr. 179 (1979) 87.
[12] A.S. Christophersen, K.E. Rasmussen, J. Chromatogr. 246 (1982) 57.
[13] M.W. Anders, G.J. Mannering, Anal. Chem. 34 (1962) 730.
[14] I. Brondz, I. Olsen, J. Chromatogr. 598 (1992) 309.
[15] J. MacGee, K.G. Allen, J. Chromatogr. 100 (1974) 35.
[16] M. Kimura, T. Yamamoto, S. Yamaguchi, Ann. Clin. Biochem. 36 (1999) 671.
[17] X.W. Fu, M. Iga, M. Kimura, S. Yamaguchi, Early Hum. Dev. 58 (2000) 41.
[18] H. van den Dool, P.D. Kratz, J. Chromatogr. 11 (1963) 463.
[19] T. Kuhara, I. Matsumoto, Proc. Jap. Soc. Biomed. Mass Spectrom. 20 (1995) 45.
[20] I. Matsumoto, T. Kuhara, Mass Spectrom. Rev. 15 (1996) 43.
[21] R. Blau, M. Duran, M.E. Blaskovics, K.M. Gibson, Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, 2nd ed., Springer, New York, 2003, p. 27.
[22] A.S. Christophersen, E. Hovland, K.E. Rasmussen, J. Chromatogr. 234 (1982) 107.
[23] M. Amijee, J. Cheung, R.J. Wells, J. Chromatogr. A 738 (1996) 43.
[24] P. Duez, A. Kumps, Y. Mardens, Clin. Chem. 42 (1996) 1609.