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Automated Metabolic Profiling and Interpretation of GC/MS Data for Organic Acidemia Screening: A Personal Computer-Based System

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Kimura, M., Yamamoto, T. and Yamaguchi, S. *Automated Metabolic Profiling and Interpretation of GC/MS Data for Organic Acidemia Screening: A Personal Computer-Based System.* Tohoku J. Exp. Med., 1999, **188** (4), 317-334 —— We have developed a personal computer-based system designed for automated metabolic profiling of urinary organic acids by gas chromatography-mass spectrometry (GC/MS) and data interpretation for organic acidemia screening. For the automated profiling, we compiled retention indices, two target ions and their intensity ratio for 126 urinary metabolites. Metabolites above the cut-off values were flagged as abnormal compounds. The data interpretation was based on combination of the flagged metabolites. Diagnostic or index metabolites were categorized into three groups, "AND," "OR" and "NO," and compiled for each disorder to improve the specificity of the diagnosis. Groups "AND" and "OR" comprised essential and optional compounds, respectively, which and both to reach a specific diagnosis. Group "NO" comprised metabolites that must be absent to make a definite diagnosis. We tested this system by analyzing urine specimens from 48 patients previously diagnosed as having organic acidemias. In all cases, the diagnostic metabolites were identified and each correct diagnosis could be found among the possible diseases suggested by the system. Hence, with this simplified automated system, more people will be able to participate extensively in any screening programs using GC/MS. —— organic acidemia; diagnostic algorithm; mass screening; GC/MS

Since gas chromatography-mass spectrometry (GC/MS) was first applied by Tanaka et al. (1966) to identify diagnostic markers in the urine from two siblings with isovaleric acidemia, this technology has been widely used for the separation of complex biological mixtures and identification of their components, and has greatly contributed to the study and characterization of a rapidly increasing

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number of organic acid disorders. The recent development of powerful and inexpensive GC/MS instruments has facilitated organic acid analysis by this method.

Mass spectrometry-based methods are now being seriously considered as a means of implementing the next generation of newborn screening programs (Millington et al. 1990; Tuchman et al. 1991; Rashed et al. 1995). In GC/MS analysis, sample preparation procedures have been improved and simplified, and several automated system for data processing have been conducted. However, there remains a need to upgrade the interpretation of results (Lehotay and Clarke 1995). For this purpose, we have developed a personal computer (PC)-based system consisting of automated metabolic profiling of urinary organic acids by GC/MS and automated data interpretation for the diagnosis or screening of organic acidemias. We describe here the principles of the system and the results of a pilot application to 48 patients with a total of 18 metabolic disorders.

Materials and Methods

Reagents

Hydroxylamine hydrochloride and margaric acid (MGA) were purchased from Wako Chemical Industries (Kyoto); the hydrocarbon mixture (C10-C26, of even numbers) and tetracosane (C24) were from Seikagaku-Kogyo Co., Ltd. (Tokyo), and N, O-bis(trimethylsilyl)-fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were from Nakarai Tesque Inc. (Tokyo).

Sample preparation

Urine samples containing 0.2 mg of creatinine were analyzed according to the previous reports with some modifications (Tanaka et al. 1980; Sweetman 1991). As internal standards, 20 μg each of MGA (Tanaka et al. 1980) and C24 were added, and the final volume was adjusted to 2.0 ml with distilled water. In the preparation, oximation was added before solvent extraction to determine 2-ketoacids as well as usual organic acids (Lancaster 1973). For oximation of 2-ketoacids, 1 ml of 5% aqueous hydroxylamine hydrochloride was added after adjusting the pH to 12–14 with 2N NaOH, followed by reaction at room temperature for 60 minutes. After acidification to pH 1.0 with 0.2 ml of 6N HCl and the addition of 1 g of NaCl, the organic acids were extracted twice with 6 ml of ethylacetate and once with 6 ml of diethylether. After centrifugation, the organic phases were combined and dehydrated with 5 g of anhydrous sodium sulfate. The supernatant was evaporated under a gentle nitrogen stream at 60°C. The final dry residue was derivatized by the addition of 100 μl of a mixture of BSTFA and TMCS (10 : 1, v : v), followed by reaction at 80°C for 30 minutes.

GC/MS analysis

A capillary GC/MS system, Shimadzu model QP 5000 (Shimadzu Co., Ltd.,

Kyoto), equipped with a class 5000 data processing system, was used. The capillary column was a fused silica DB-5 one (30 m×0.25 mm i.d.) with a 1 μm film thickness of 5% phenylmethyl silicone (J & W, Folsom, CA, USA). Mass spectra were obtained by standard electron impact ionization scanning from m/z 50 to m/z 600 at the rate of 0.4 seconds/cycle.

The temperature program was started at 100°C with initial holding for 4 minutes, and was increased at the rate of 4°C/minutes to 290°C, with final holding for 10 minutes. The temperatures of the injection port and transfer line were both 280°C. The flow rate of the helium carrier was 1.5 ml/minutes, and the linear velocity was 40.2 m/second. One μl of the final derivatized aliquot was injected into the GC/MS in the splitless mode.

Patient samples

Urine samples from 48 patients with 18 kinds of organic acidemias (previously diagnosed) were analyzed to evaluate the reliability of the diagnostic algorithms of this system. The disorders and the respective numbers of patients are listed in Table 1. Moreover, urine samples from a healthy control and a patient with propionic acidemia were analyzed 10 times each to test the reproducibility of the system.

TABLE 1. *Results of analysis of the 48 patients tested in this study*

	Disease	*n*	Age	Abnormal compounds detected	Suspected disease
1	Methylmalonic acidemia	7	2 days-2 years	MMA (7), MC (7), 3HP (5), HMG (3), GA (3), LA (5), 3HB (6), AD (3), PG (3), PHPLA (3)	MMA-emia (7), KET (6), LA-emia (5), DCA (3)
2	Propionic acidemia	4	1 week-2 years	3HP (3), PG (4), TG (3), MC (4), 3HB (2), LA (2)	PA-emia (4), LA-emia (2), KET (2)
3	3-Ketothiolase deficiency	1	10 years	2M3HB, TG	3-Ketothiolase deficiency
4	Isovaleric acidemia	3	11 days-5 months	IVG (3), 3HIV (2), AD (1)	Isovaleric acidemia (3), DCA (1)
5	3-Methylcrotonyl-glycinuria	1	17 years	3MCG, 3HIV	3-Methylcrotonyl-glycinuria
6	3-Methylglutaconic aciduria	1	1 year	LA, PYR, 3MG	3-Methylglutaconic aciduria, LA-emia
7	3-Hydroxy-3-methyl-glutaric aciduria	2	7 months, 5 years	MGC (2), MG (2), HMG (2)	HMG-uria (2)
8	Multiple carboxy-lase deficiency	2	1 day, 6 months	3MCG (2), MC (2), 3HIV (2), LA(1), PYR (1), PG (1), 3HP (1), 3HB (1), 2KIC (1), 2HIV (1)	MCD (2), KET (1), LA-emia (1), MSUD (1)
9	Glutaric aciduria type I	3	5 months, 1 year	GA (3), 3HG (3), AD (2), SUB (2), 3HB (1), AA (1), SEB (1), 3SU (1), 3SE (1), 3HDD (1)	GA1 (3), KET (2), DCA (2), 3HDCA (1)

TABLE 1. *(Continued)*

	Disease	*n*	Age	Abnormal compounds detected	Suspected disease
10	Glutaric aciduria type II	4	1 month-1 year	Ethymalonic (4), IVG (4), 2HG (3), AD (2), SUB (2), SEB (2)	GA2 (4), DCA (2)
11	2-Hydroxyglutaric aciduria	1	5 years	2HG	2-Hydroxyglutaric aciduria
12	L-Glyceric aciduria	1	10 months	Glyceric acid	Glyceric aciduria
13	5-Oxoprolinuria	2	7 days, 10 years	5-Oxoproline (2), LA (2), PYR (2)	5-Oxoprolinuria (2), LA-emia (2)
14	3-Hydroxyisobutyric aciduria	1	1 year	3-Hydroxyisobutyric	3-Hydroxyisobutyric aciduria
15	Ornithine transcarbamylase deficiency	7	4 days-6 years	ORO (7), uracil (5), 3HB (5), AA (3), glycerol (2)	OTCD (7), LA-emia (2), KET (6), glyceroluria (2)
16	Tyrosinemia type I	2	4 months, 5 months	SA (2), PHPLA (2), PHPPA (2), LA (1), 3HB (1)	Tyrosinemia 1 (2), KET (1)
17	Maple syrup urine disease	1	12 days	2HIV, 2HIC, 2KIC, 2K3MV, LA, 3HB	MSUD, LA-emia, KET
18	Glyceroluria	5	5 days-4 years	Glycerol (5)	Glyceroluria (5)

The abbreviations are as follows: AA, acetoacetic acid; AD, adipic acid; DCA, dicarboxylic aciduria; GA, glutaric acid; GA1, glutaric aciduria type 1; GA2, glutaric aciduria type 2; 3HB, 3-hydroxybutyric acid; 3HDD, 3-hydroxydodecanedioic acid; 3HDCA, 3-hydroxydicarboxylic aciduria; 2HG, 2-hydroxyglutaric acid; 3HG, 3-hydroxyglutaric acid; 2HIC, 2-hydroxyisocaproic acid; 3HIV, 3-hydroxyisovaleric acid; HMG, 3-hydroxy-3-methylglutaric acid; HMG-uria, 3-hydroxy-3-methylglutaric aciduria; 3HP, 3-hydroxypropionic acid; 3HSE, 3-hydroxysebacic acid; 3HSU, 3-hydroxysuberic acid; IVG, isovalerylglycine; KET, ketosis; 2KIC, 2-ketoisocaproic acid; 2K3MV, 2-keto-3-methylvaleric acid; LA, lactic acid; LA-emia, lactic acidemia; MC, methylcitric acid; MCD, multiple carboxylase deficiency; MCG, 3-methylcrotonylglycine; MGA, 3-methylglutaric acid; MGC, 3-methylglutaconic acid; 2M3HB, 2-methyl-3-hydroxybutyric acid; MMA, methylmalonic acid; MMA-emia, methylmalonic acidemia; MSUD, maple syrup urine disease; ORO, orotic acid; PA-emia, propionic acidemia; PG, propionylglycine; PHPLA, p-hydroxyphenyllactic acid; PHPPA, p-hydroxyphenylpyruvic acid; PYR, pyruvic acid; SA, succinylacetone; SEB, sebacic acid; SUB, suberic acid; TG, tiglylglycine.

The numbers in parentheses following the compounds or disease names indicate the numbers of patients.

Automated metabolic profiling system

The personal computer used for this system was a COMPAQ DESKPRO 2000 5200, running Microsoft Windows 3.1. Prior to GC/MS analysis of urine samples, an analysis of an even-numbered hydrocarbon mixture (C10 to C26) was performed daily. The retention times of individual hydrocarbon species were recorded

with the system, and using this data the predicted retention times of individual compounds were calculated according to the methylene unit (MU) values (Tanaka et al. 1980).

The identification of a compound with this program was based on the following three parameters: 1) The co-elution of two target ion species on mass chromatogram, one for quantification (Q-ion) and the other for confirmation (C-ion), chosen to maximize the differences from other closely eluting compounds; 2) the ratio of their peak intensities (C/Q ratio) for further specification; and 3) the retention time calculated from each MU value. Values higher than 50% of the C/Q ratio, calculated from the mass spectra of the authentic standards, were designated as the C/Q ratios for individual compounds in this system. The retention times of each peak on mass chromatograms was confined within a 2% width window of the predicted retention times. The accuracy of the retention times on a total ion current chromatogram (TIC) was conveniently checked by comparing the retention times of C24 in the hydrocarbon mixture and added to the urine samples.

As an example, the TIC and mass chromatograms of several compounds in the urine sample from a patient with propionic acidemia are illustrated in Fig. 1. The top 4 traces (m/z 99, m/z 67, m/z 327, and m/z 145) are of the Q- and C-ions of two internal standards, C24 and MGA. The bottom 6 ions are the Q- and C-ions of the diagnostic markers, methylcitrate (MC), propionylglycine (PG), and 3-hydroxypropionic acid (3HP). When a metabolite was identified as described above, semi-quantitative determination was performed by calculation of the relative peak area (RPA, %) as to the Q-ions of the compound and the internal standard (Q-ion of MGA, m/z 327).

The mean values, standard deviations (S.D.), and RPA ranges (%) in normal controls for individual metabolites were determined. The normal tables for several age groups, neonates (around 5-day-old newborns), infants (1- to 3-month-old babies), young children (1 to 3 year-old children), and school-age-children, were prepared, analyzing at least 30 normal individuals for each age group. The mean plus 5 S.D. values for the normal controls for each age group were basically designated as the cut-off values. In the case of metabolites which were usually undetectable in the normal controls, the RPA value of 0.1% as the peak area ratio was conveniently designated as the cut-off. Our experience suggested that this is a minimum value that can be distinguished from baseline artifacts on GC/MS. If the obtained value exceeded the cut-off level, the compound was flagged with an asterisk next to the RPA value indicating that it was potentially abnormal.

An additional feature of the program is the ability to printout the TIC. In this TIC, identified peaks were filled in with black with their identification numbers at the top. Furthermore, peaks of abnormal metabolites were flagged with asterisks next to the identification numbers. It may be necessary to check

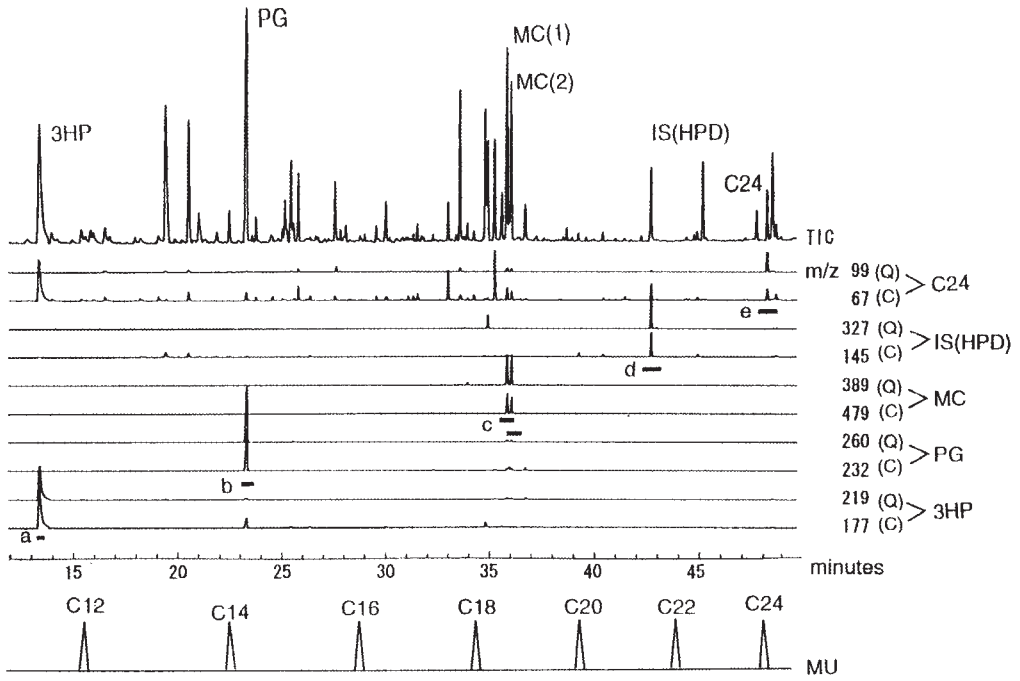

Fig. 1. Total ion current chromatogram (TIC) and mass chromatograms of gas chromatography/mass spectrometry (GC/MS) analysis of a patient with propionic acidemia: Principles of automated metabolic profiling.

The abbreviations are as follows: 3HP, 3-hydroxypropionic acid; PG, propionylglycine; methylcitrate (MC) (1) and MC (2), the first and second peaks of methylcitric acid, respectively; tetracosane (C24,); IS, margaric acid (MGA), as an internal standard; C10, C12, C14, ... C26, the respective carbon numbers of the even-numbered hydrocarbon mixture. Q and C, the Q- and C-ions, respectively; MU, methylene unit value.

A metabolite is identified when the following three conditions co-exist: 1) the co-elution of two target ions, one for quantitation (Q-ion) and the other for confirmation (C-ion); 2) an appropriate ratio of their peak intensities (C/Q ratio, see the text); and 3) the retention times on mass chromatograms confined within a 2% width window of the predicted retention time calculated from the MU values. The short bold bars below peaks a, b, c, d and e indicate the predicted retention times and width windows for 3HP, PG, MC, MGA and C24, respectively.

the mass spectra manually before the final diagnosis, of flagged or unfilled peaks found on the TIC. There is a possibility that they are either unknown pathological metabolites or other compounds derived from exogenous factors, such as diet, or medication.

Automated data interpretation system

Table 2 is a list of the 25 disorders covered by this system at present, and the metabolites used for diagnostic purposes. The metabolites were classified into the following three categories, namely "AND," "OR" and "NO," for the data interpretation system. Categories "AND" and "OR" comprised essential and optional compounds required to reach a specific diagnosis, respectively. The third category, "NO," comprised compounds that must be absent to make a definite diagno-

TABLE 2. *Disease and index metabolites compiled in the data interpretation system*

No.	Disease	And	Or	No
1	Methylmalonic acidemia	Methylmalonic		
2	Propionic acidemia		Propionylglycine Methylcitric	Methylmalonic 3-Methylcrotonylglycine
3	3-Ketothiolase deficiency	2-Methyl-3-hydroxybutyric Tiglylglycine		Methylmalonic Propionylglycine Methylcitric
4	Isovaleric acidemia	Isovalerylglycine		Methylmalonic Propionylglycine 2-Hydroxyglutaric Methylcitric
5	3-Methylcrotonylglycinuria	Methylcrotonylglycine		Methylcitric Propionylglycine
6	3-Methylglutaconic aciduria	3-Methylglutaconic		3-Hydroxy-3-methylglutaric
7	3-Hydroxy-3-methylglutaric aciduria	3-Hydroxy-3-methylglutaric		3-Hydroxybutyric
8	Multiple carboxylase deficiency	Methylcrotonylglycine Methylcitric		Methylmalonic
9	Glutaric aciduria type I	Glutaric 3-Hydroxyglutaric		Methylmalonic Propionylglycine
10	Glutaric aciduria type II	Ethylmalonic Isovalerylglycine 2-Hydroxyglutaric		
11	2-Hydroxyglutaric acidemia	2-Hydroxyglutaric		Isovalerylglycine Ethylmalonic
12	2-Ketoadipic aciduria	2-Ketoadipic		
13	L-Glyceric aciduria	Glyceric		
14	5-Oxoprolinuria	5-Oxoproline		
15	3-Hydroxyisobutyric aciduria	3-Hydroxy-isobutyric		
16	Ornithine transcarbamylase deficiency suspected		Orotic Uracil	
17	Tyrosinemia type I	Succinylacetone 4-Hydroxyphenyllactic		

TABLE 2. *(Continued)*

No.	Disease	And	Or	No
18	Alcaptonuria	Homogentistic		
19	Maple syrup urine disease	2-Hydroxyisovaleric 2-Keto-isocaproic		
20	Glyceroluria suspected	Glycerol		
21	Mevalonic acidemia	Mevalonolactone		
22	Lactic acidemia (condition)		Lactic Pyruvic	
23	Ketosis (condition)		3-hydroxybutyric Acetoacetic	
24	Dicarboxylic acidemia (condition)		Adipic Suberic Sebacic Dodecanedioic	
25	3-Hydroxy-dicarboxylic acidemia (condition)		3-Hydroxyadipic 3-Hydroxysuberic 3-Hydroxysebacic 3-Hydroxy-dodeca-nedioic	

AND, metabolites which are indispensable for a diagnosis; OR, metabolites of which some are essential for a diagnosis; NO, metabolites which must be absent to make a diagnosis.

sis. For example, the absence of methylmalonic acid is required for the diagnosis of propionic acidemia or 3-ketothiolase deficiency, as illustrated in the metabolic map (Fig. 2).

RESULTS

Compiling the GC/MS data for the system

The parameters for peak identification, including the Q and C-ions, C/Q ratio, and MU values for 126 compounds, were compiled according to the previous reports (Tanaka et al. 1980; Sweetman 1991), or from our data obtained by analysis of authentic compounds or urine samples from patients. The MU values for the 127 compounds including C24 are listed in Table 3. These compounds include intrinsic or extrinsic metabolites that were commonly observed in urine, or related to well-recognized organic acid disorders or other pathological conditions. The number of compounds can be increased as the need arises in the future.

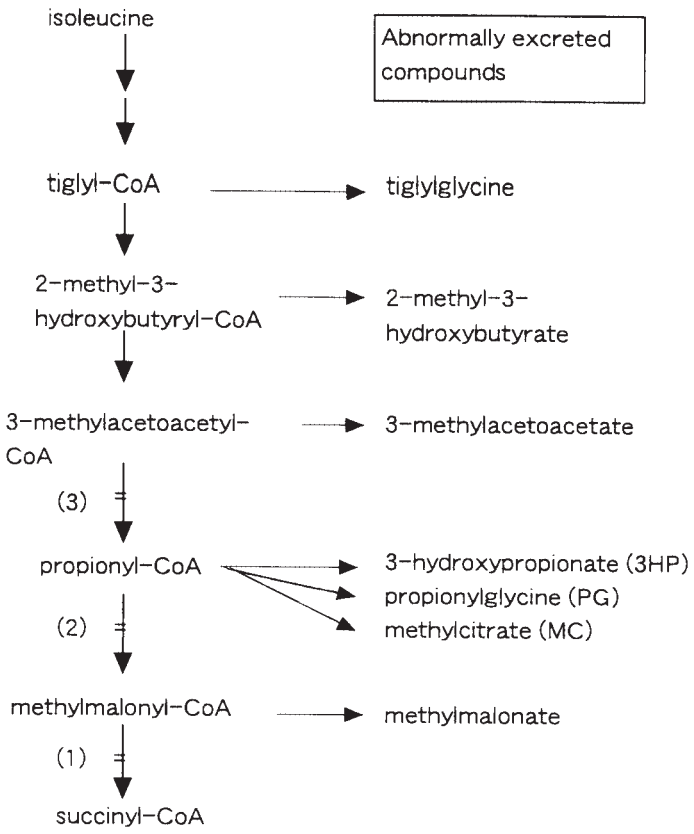

Fig. 2. Metabolic map of isoleucine catabolism related to propionic acidemia. (1), (2) and (3) indicate the metabolic block sites in methylmalonic acidemia, propionic acidemia and 3-ketothiolase deficiency. In the left part, abnormally excreted metabolites in urine corresponding to the respective intermediates accumulated. For example, in propionic acidemia, caused by the block of the propionyl-CoA carboxylase site (2), 3-hydroxypropionic acid, propionylglycine or methylcitric acid derived from accumulated propionyl-CoA, are increased, despite no elevation of methylmalonic acid.

Testing the usefulness of this system

To test the accuracy of this system, urine samples from a total of 48 patients with 18 kinds of organic acidemias, as shown in Table 1, were analyzed, and the data were processed by this system. In all cases, at least one indicative metabolite was automatically flagged, and a correct diagnosis was found among the possible diseases suggested by this system. Each data processing took less than a minute.

Furthermore, we tested the accuracy or reproducibility of the semi-quantification in the system, separately preparing and analyzing urine samples from a healthy control and a patient with propionic acidemia 10 times each, as shown in Table 4. Uracil, 3HP, 3-hydroxybutyric acid (3HB) and MC exhibited great variability with the coefficient of variation greater than 30%. The data interpretation system suggested the following possible diseases, none for all the specimens from the healthy control, and "propionic acidemia" in all the specimens

TABLE 3. *List of 126 compounds preenrolled in this system and their MU values on DB-5 capillary column*

No.	Compound	MU	No.	Compound	MU
1	Lactic-2	10.57	41	Propionylglycine-1	13.59
2	2-Hydroxyisobutyric-2	10.69	42	Mevalonolactone-2	13.88
3	Glycolic-2	10.78	43	Mevalonolactone-1	13.93
4	Oxalic-2	11.31	44	Isobutyrylglycine-1	13.92
5	2-Hydroxybutyric-2	11.32	45	2-Propyl-3-hydroxypentanoic-2(VPA)	13.93
6	Glyoxylic-oX-2	11.35	46	Mesaconic-2	14.00
7	3-Hydroxypropionic-2	11.44	47	Glutaric-2	14.04
8	Pyruvic-OX-2	11.49	48	3-Methylglutaconic-2	14.17
9	Vaproic-1	11.50	49	3-Methylglutaric-2	14.26
10	3-hydroxybutyric-2	11.63	50	2-Propyl-3-ketopentanoic-2(VPA)	14.26
11	3-Hydroxyisobutyric-2	11.64	51	Propionylglycine-2	14.28
12	2-Hydroxyisovaleric-2	11.71	52	Isobutyrylglycine-2	14.30
13	2-Methyl-3-hydroxybutyric-2	12.02	53	2-Deoxytetronic-3	14.39
14	Melonic-2	12.05	54	Butyrylglycine-1	14.42
15	3-Hydroxyisovaleric-2	12.14	55	3-Methylglutaconic-2	14.44
16	2-keto-isovaleric-OX-2	12.14	56	Glutaconic-2	14.48
17	Methylmalonic-2	12.19	57	Succinylacetone-OX-2	14.53
18	Ethylhydracrylic-2	12.32	58	2-Propyl-5-hydroxypentanoic-2(VPA)	14.84
19	Urea-2	12.37	59	3-Methylglutaconic-2	14.84
20	4-Hydroxybutyric-2	12.38	60	Isovalerylglycine-1	14.88
21	2-Hydroxyisocaproic-2	12.42	61	Butyrylglycine-2	14.92
22	3-Hydroxyvaleric-2	12.42	62	Malic-3	14.99
23	Acetoacetic-2	12.49	63	Adipic-2	15.10
24	2-Hydroxy3-methylvaleric-2	12.50	64	Isovalerylglycine-2	15.20
25	Benzoic-1	12.53	65	2-Hexenedioic-2	15.22
26	Acetoacetic-OX-2	12.61	66	5-Oxyproline-2	15.34
27	2-Keto-3-methylvaleric-OX-2	12.76	67	3-Methyladipic-2	15.39
28	2-Methyl-3-hydroxyvaleric-2 (1)	12.76	68	Thiodiglycolic-2	15.41
29	Glycerol-3	12.82	69	2-Propylglutaric-2 (VPA)	15.51
30	Phosphoric-3	12.83	70	7-Hydroxyoctanoic-2	15.51
31	2-Methyl-3-hydroxyvaleric-2 (2)	12.84	71	5-Hydroxymethyl-2-furoic-1	15.54
32	Ethylmalonic-2	12.86	72	Tiglylglycine-2	15.64
33	2-Keto-isocaproic-OX-2	12.91	73	3-Methylcrotonylglycine-1	15.64
34	Phenylacetic-1	13.03	74	Tiglylglycine-1	15.71
35	Maleic-2	13.10	75	3-Methylcrotonylglycine-2	15.78
36	Succinic-2	13.16	76	2-Hydroxyglutaric-3	15.81
37	Methylsuccinic-2	13.28	77	3-Hydroxyglutaric-3	15.82
38	Glyceric-2	13.42	78	Phenyllactic-2	16.00
39	Uracil-2	13.46	79	Pimelic-2	16.04
40	Fumaric-2	13.48	80	3-Hydroxy-3-methylglutaric-3	16.11

81	3-Hydroxyphenylacetic-2	16.16	105	Methylcitric-4 (1)	18.62
82	2-Ketoglutaric-OX-2 (1)	16.35	106	3-(3-Hydroxy-phenyl)-3-hydroxypropionic-3	18.64
83	4-Hydroxybenzoic-2	16.37	107	Methylcitric-4 (2)	18.71
84	4-Hydorxyphenylacetic-2	16.48	108	3-Hydroxysuberic-3	18.81
85	2-Ketoglutaric-OX-2 (2)	16.55	109	Vanilmandelic-3 (VMA)	18.96
86	Hexanoylglycine-1	16.55	110	Sebacic-2	18.99
87	Phenylpyruvic-OX-2	16.62	111	Decadienedioic-2	19.03
88	N-Acetylaspartic-2	16.71	112	4-Hydroxyphenyllactic-2 (PHPLA)	19.19
89	2-Hydroxyadipic-3	16.79	113	4-Hydroxyphenylpyruvic-OX-2 (PHPPA)	19.51
90	Octanedioic-2	16.82	114	2-Hydroxyhippuric-2	19.73
91	3-Hydroxyadipic-3	16.91	115	Indole-3-acetic-2	19.84
92	Suberic-2	17.02	116	Suberylglycine-2	20.26
93	3-Methylglutaconic-2	17.14	117	Palmitic-1	20.47
94	2-Keto-adipic-OX-3	17.17	118	2-Hydroxysebacic-3	20.59
95	Aconitic-3	17.54	119	3-Hydroxysebacic-3	20.71
96	Orotic-3	17.58	120	2-Hydroxyhippuric-2	20.86
97	Vanillic-2	17.72	121	Dodecanedioic-2	20.89
98	Homovanillic-2 (HVA)	17.83	122	N-acetyltyrosine-3	21.19
99	Azelaic-2	18.00	123	Uric-4	21.27
100	Hippuric-2	18.20	124	3,6-Epoxydodecanedioic-2	21.71
101	Isocitric-4	18.37	125	3-Hydroxydodecanedioic-3	22.62
102	Citric-4	18.38	126	3,6-Epoxytetradecanedioic-2	23.61
103	Homogentistic-3	18.50	127	Tetracosane (C24)	24.00
104	Hippuric-1	18.51			

The numbers next to the compound names represent as follows:

Lactic-2 or Benzoic-1, the number indicates trimethylsilyl (TMS) groups, namely lactic acid diTMS or benzoic acid monoTMS, respectively; pyruvic-OX-2, oximated pyruvic acid diTMS; 2-Ketoglutaric-OX-2 (1), the first of two peaks of oximated 2-ketoglutaric acid diTMS.

and "ketosis" in 9 of the 10 specimens from the patient with propionic acidemia.

Examples of cases of propionic acidemia and multiple carboxylase deficiency which were processed with this system are presented in Tables 5 and 6, respectively. These tables show the data obtained with automated metabolic profiling and data interpretation after GC/MS analysis.

Propionic acidemia is caused by a deficiency of propionyl-CoA carboxylase in the intermediate catabolism of isoleucine, valine, threonine or methionine (Fenton and Rosenberg 1995). In Fig. 2, one of the catabolism pathway, that from isoleucine is shown. In the urinary organic acid profile of such patients, 3HP, PG and MC, which are derived from accumulated propionyl-CoA, are elevated. Multiple carboxylase deficiency 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-

TABLE 4. *Reproducibility of semi-quantification*

Sample	No.	Compound	Mean (%)	S.D.	CV
1	4	Oxalic-2	13.22	1.97	0.15
	8	Pyruvic-OX-2	8.97	2.29	0.26
	36	Succinic-2	28.02	2.50	0.09
	39	Uracil-2	1.99	0.67	0.34
	40	Fumaric-2	3.74	0.36	0.10
	66	5-Oxoproline-2	5.31	0.84	0.16
	76	2-Hydroxyglutaric-3	2.16	0.16	0.07
	82	2-Ketoglutaric-OX-2 (1)	18.22	0.55	0.03
	95	Aconitic-3	95.90	2.93	0.03
	109	Vanilmandelic-3 (VMA)	53.67	3.86	0.07
	112	4-Hydroxyphenyllactic-2 (PHPLA)	14.95	0.80	0.05
2	7	3-Hydroxypropionic-2	99.74	46.16	0.46
	10	3-Hydroxybutyric-2	11.66	3.51	0.30
	41	Propionylglycine-1	46.14	8.06	0.17
	51	Propionylglycine-2	7.24	1.80	0.25
	63	Adipic-2	20.83	1.19	0.06
	74	Tiglylglycine-1	18.00	1.60	0.09
	80	3-Hydroxy-3-methylglutaric-3	37.36	6.23	0.17
	105	Methylcitric-4 (1)	15.52	3.60	0.23
	107	Methylcitric-4 (2)	8.92	2.73	0.31

Means, standard deviation (S.D.) and coefficients of variation of relative peak areas (%) of the Q-ions of the compounds and the internal standard (MGA, Q-ion m/z 327). Samples 1 and 2, were from a healthy control and a patient with propionic acidemia respectively. The two samples were separately prepared and analyzed 10 times. The GC/MS data were processed by the automated metabolic profiling and data interpretation.

CoA carboxylase. Urinary organic acid analysis of such patients reveals elevation of not only 3HP, PG, and MC diagnostic of propionic acidemia, but also 3-methylcrotonylglycine (MCG) and 3-hydroxyisovalerate (3HIV), diagnostic of 3-methylcrotonyl-CoA carboxylase deficiency.

In Table 5, urinary metabolites are profiled, and several abnormal compounds, such as 3HP, 3HB, 2-methyl-3-hydroxybutyrate, 3HIV, PG, adipate, and MC, are indicated by asterisks. Subsequently, the suspected diagnosis should be "propionic acidemia" as well as "ketosis" and "dicarboxylic aciduria" are listed. Hence, we could deduce that this patient had propionic acidemia associated with ketotic dicarboxylic aciduria, probably in an acute condition.

On the other hand, in the metabolic profile of another patient, as shown in Table 6, not only 3HP, PG and MC which are diagnostic of propionic acidemia, but also 3HIV and MCG, which are diagnostic of 3-methylcrotonyl-CoA carbox-

TABLE 5. *Results of the case of propionic acidemia patient processed by the automated metabolic profiling and data interpretation system (the data are partly shown)*

ID	Compound	Value	Normal	Range	Factor
1	Lactic-2	2.5404	0.80	(0.00–4.70)	3.18
7	3-Hydroxypropionic-2	320.2869*	0.20	(0.00–1.10)	1601.43
10	3-Hydroxybutyric-2	18.2578*	0.70	(0.00–3.70)	26.08
11	3-Hydroxyisobutyric-2	2.6775	2.50	(0.00–9.00)	1.07
13	2-Methyl-3-hydroxybutyric-2	3.9699*	0.05	(0.00–0.30)	?
15	3-Hydroxyisovaleric-2	7.3928*	0.80	(0.00–2.30)	9.24
17	Methylmalonic-2	3.7970	0.35	(0.00–3.60)	12.66
19	Urea-2	58.3530	376.10	(0.00–763.0)	0.16
41	Propionylglycine-1	40.1994*	0.00	(0.00–0.00)	?
46	Mesaconic-2	0.9683	1.50	(0.00–8.90)	0.65
47	Glutaric-2	28.2624*	1.90	(0.00–4.00)	14.87
51	Propionylglycine-2	287.9753*	0.00	(0.00–0.00)	?
52	Isobutyrylglycine-2	9.5286*	0.00	(0.00–0.00)	?
62	Malic-3	10.7793*	0.10	(0.00–0.70)	107.79
63	Adipic-2	25.8676*	3.00	(0.00–5.00)	8.62
64	Isovalerylglycine-2	2.7501*	0.00	(0.00–0.00)	?
66	5-Oxoproline-2	3.1697	0.90	(0.00–7.60)	3.52
101	Isocitric-4	11.3585	22.90	(8.30–29.00)	0.50
102	Citric-4	329.7592	441.10	(31.40–572.30)	0.75
104	Hippuric-1	19.2495	30.10	(6.20–284.10)	0.64
105	Methylcitric-4 (1)	103.1942*	0.20	(0.00–1.10)	515.97
107	Methylcitric-4 (2)	75.4122*	0.10	(0.00–1.00)	754.12
108	3-Hydroxysuberic-3	8.8933	1.20	(0.00–4.83)	7.41
109	Vanilmandelic-3 (VMA)	49.3502	46.60	(11.70–84.60)	1.06
110	Sebacic-2	4.4764	2.20	(0.40–7.00)	2.03

No.	Interpretation:
2	Propionic acidemia
23	Ketosis
24	Dicarboxylic aciduria

The upper part shows a metabolic profile. Compounds judged abnormal are indicated with asterisks next to the values. Value, relative peak area (%) of the Q-ions of the compound and the internal standard (MGA, Q-ion m/z 327); Normal, mean for normal controls (30 healthy children aged 3 months); Range, minimum and maximum values in normal controls; Factor, number of times the mean value; asterisk (*) indicated abnormal, over the cut-off values.

In the bottom part, suspicious diagnosis or pathological conditions are suggested from the data interpretation system. The compound names and abbreviations are the same as those indicated in Table 3.

TABLE 6. *Results of the case of multiple carboxylase deficiency patient (the data are partly shown)*

ID	Compound	Value	Normal	Range	Factor
1	Lactic-2	4.4224	0.80	(0.00–4.70)	5.33
5	2-Hydroxybutyric-2	0.4019	0.00	(0.00–0.00)	?
7	3-Hydroxypropionic-2	1.9971*	0.20	(0.00–1.10)	9.99
8	Pyruvic-OX-2	6.0800	4.50	(0.00–24.10)	1.35
10	3-hydroxybutyric-2	4.7738	0.70	(0.00–3.70)	6.82
11	3-Hydroxyisobutyric-2	2.5585	2.50	(0.00–9.00)	1.02
15	3-Hydroxyisovaleric-2	116.1751*	0.80	(0.00–2.30)	145.22
18	Ethylhydracrylic-2	2.0481	0.00	(0.00–2.90)	?
19	Urea-2	106.4862	376.10	(0.00–763.0)	0.28
51	Propionylglycine-2	0.6806*	0.00	(0.00–0.00)	?
53	2-Deoxytetronic-3	1.6248	2.40	(0.00–6.30)	0.68
55	3-Methylglutaconic-2	1.7013	1.10	(0.00–4.20)	1.55
56	Glutaconic-2	0.3204	0.00	(0.00–0.00)	?
59	3-Methylglutaconic-2	1.5359	1.50	(0.00–2.90)	1.02
62	Malic-3	0.8055	0.10	(0.00–0.70)	8.06
63	Adipic-2	0.8653	3.00	(0.00–5.00)	0.29
64	Isovalerylglycine-2	0.6747*	0.00	(0.00–0.00)	?
66	5-Oxoproline-2	2.4216	0.90	(0.00–7.60)	2.69
67	3-Methyladipic-2	3.3282	4.30	(0.00–23.3)	0.77
68	Thiodiglycolic-2	5.2606*	0.00	(0.00–0.00)	?
71	5-Hydroxymethyl-2-furoic-1	3.7745	0.00	(0.00–0.00)	?
73	3-Methylcrotonylglycine-1	5.9214*	0.00	(0.00–0.00)	?
75	3-Methylcrotonylglycine-2	133.3985*	0.00	(0.00–0.00)	?
76	2-Hydroxyglutaric-3	1.3112	2.30	(0.60–5.90)	0.57
78	Phenyllactic-2	0.4899	0.30	(0.00–4.90)	1.63
79	Pimelic-2	3.5304	3.00	(0.00–9.30)	1.18
80	3-Hydroxy-3-methylglutaric-3	3.6889	2.90	(0.00–25.70)	1.27
101	Isocitric-4	13.4793	22.90	(8.30–29.00)	0.59
102	Citric-4	179.0200	441.10	(31.40–572.30)	0.41
104	Hippuric-1	36.6646	30.10	(6.20–284.10)	1.22
105	Methylcitric-4 (1)	3.1433*	0.20	(0.00–1.10)	15.72
107	Methylcitric-4 (2)	2.9508*	0.10	(0.00–1.00)	29.51
109	Vanilmandelic-3 (VMA)	97.2244	46.60	(11.70–84.60)	2.09

No.	Interpretation:
8	multiple carboxylase deficiency

Formats and abbreviations are the same as in Table 5. The data in the upper part of metabolic profile are partly shown. In the bottom part, only a disease, multiple carboxylase deficiency is indicated.

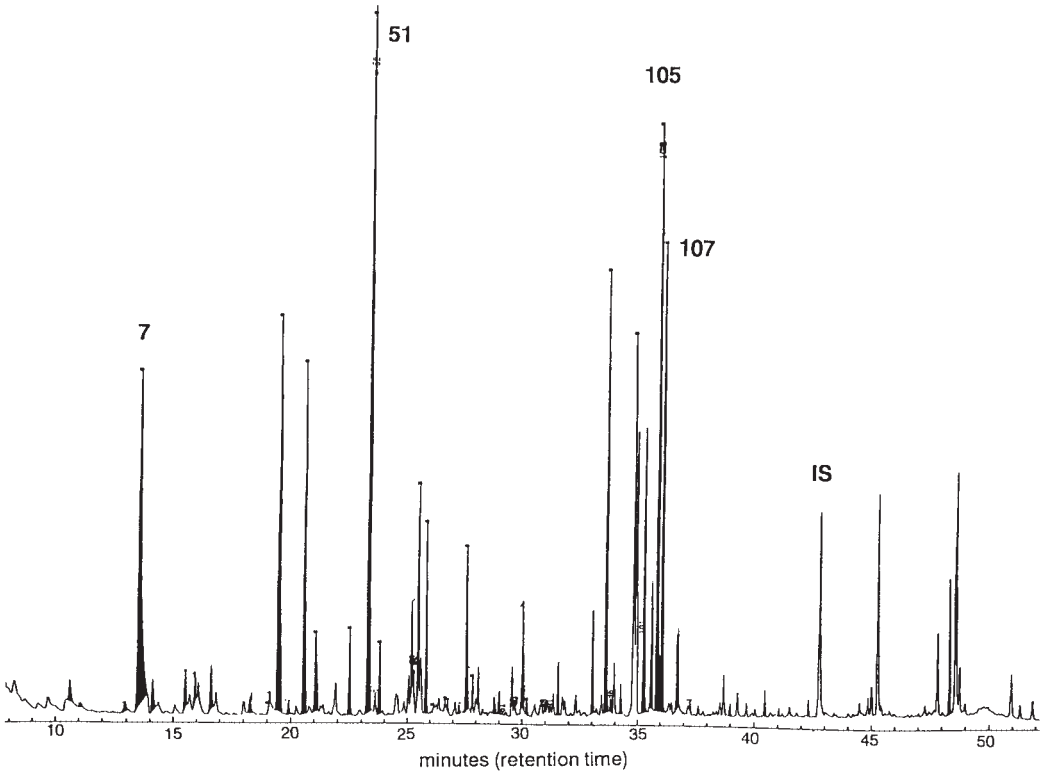

Fig. 3. The total ion current chromatogram (TIC) of this system on a case of propionic acidemia.

The same GC/MS analysis data of this patient is shown in Table 5. The peaks identified are filled in black. The peak identification numbers are marked at the top of each peak. Peaks judged abnormal are indicated with asterisks at the peak tops. Peaks 7, 51, 105 and 107, which are marked with asterisks represent 3-hydroxypropionic acid, porpionylglycine, methylcitric acid peak 1 and peak 2, respectively. IS indicates an internal standard. Peaks unidentified in this system are not filled in black.

ylase deficiency, were flagged simultaneously. The elevation of 3HIV was striking. "Multiple carboxylase" was indicated in the bottom. Hence, we could deduce that this patient has multiple carboxylase deficiency, but has neither propionic acidemia nor isolated 3-methylcrotonyl-CoA carboxylase deficiency.

In the TIC with this system, as shown in Fig. 3, peaks identified were filled in with black. The identification numbers were given at the tops of the peaks, with asterisks for peaks judged abnormal. Several mass spectrum of peaks flagged with asterisks or unidentified could be confirmed on this TIC, and was able to reach a final diagnosis.

Several pathological conditions other than defined disorders, such as dicarboxylic aciduria, lactic acidemia, ketosis, and valproate intake, were also well identified. As illustrated in Table 4, "ketosis" and "dicarboxylic aciduria" as well as "propionic acidemia" were listed. These gave not only the specific diagnosis, propionic acidemia, but also other complicating conditions in this patient. Several diseases whose diagnoses can not be definitely confirmed by organic acid analysis alone, such as maple syrup urine disease, tyrosinemia type I,

ornithine transcarbamylase deficiency, or glyceroluria, were also indicated. Hence, this system gave accurate information for all patients examined in this study.

Discussion

Recently, a bench top GC/MS with a capillary column, which is compact, easy to maintain and less expensive, has been developed. Furthermore, powerful, highly-efficient and user-friendly personal computers and software have also been developed. These advances make GC/MS popular in general clinical laboratories. However, automated interpretation of GC/MS data is a complex task that has not been perfected (Lehotay and Clarke 1995), although automated data profiling has been developed in several laboratories (Gates and Sweely 1978; Gates et al. 1978; Sweetman 1991). GC/MS data interpretation requires consideration of the entire profile of organic acids as well as other factors like the relative amounts of each organic acid, the age of the patients, or of variables due to drug exposure, diet, and the clinical conditions at sampling.

We have developed a PC-based system for automated interpretation of the GC/MS data as well as automated metabolic profiling of urinary organic acids. On the analysis of urinary samples from 48 patients with 18 different metabolic diseases, it was confirmed that our system exhibited usefulness in the diagnosis of diseases or pathological conditions. The principle of automated metabolic profiling in our system is basically similar to those previously reported in terms of peak identification. They were based upon a reverse library search of target ions in the region of the expected retention time of each compound of interest. In our system, unknown or unidentified peaks as well as abnormal peaks were easily detected on TIC chromatogram. The most unique feature in our system is a newly developed "automated data interpretation system." It is a diagnostic program by means of a combination of diagnostic markers, with the categorization of indicative metabolites into "AND," "OR," and "NO." It allowed us to reach a specific diagnosis or perform metabolic evaluation.

Several non-specific conditions such as lactic acidemia, ketosis and dicarboxylic aciduria were also included in the data interpretation system. They may help to evaluate a patient's condition at sampling. Furthermore, our system may suggest disorders other than defined organic acidemias, like Zellweger syndrome, according to the organic acid profiles.

To increase the specificity of this data interpretation system, further alterations in the cut-off points of marker metabolites or compound names categorized might be needed. In our pilot tests on 48 patients, however, there were not any serious misinterpretations of data. For example, in cases of propionic acidemia and multiple carboxylase deficiency as shown in Tables 5 and 6, isovalerylglycine, a diagnostic marker metabolite of isovaleric acidemia, was flagged as one of the abnormal metabolites. The reason why the disease name of isovaleric acidemia

was not found among the suspected disease names is that the "No" category of isovaleric acidemia includes methylmalonic acid, PG, 2-hydroxyglutaric acid, and MC as shown in Table 2.

We also showed the limitations of semi-quatification using a single internal standard, MGA, in this study. Several metabolites, such as uracil, 3HB, and MC, exhibited considerable variation on repeated analysis of the same sample. This variability may not be due to a disadvantage of the system, but rather to the method of solvent extraction and the adoption of a single internal standard. When GC/MS analysis requires high sensitivity and reproducibility, such as mass screening, appropriate internal standards such as stable isotope-labeled compounds should be used. Multiple internal standards can also be designated to respective compounds with this system. It is applicable for other analyses such as the stable isotope dilution method or simultaneous GC/MS analysis of organic acids, amino acids, carbohydrates and polyols (Schoemaker and Elliot 1991; Matsumoto and Kuhara 1996).

Recently, the neonatal mass screening of organic acidemias by means of GC/MS or tandem mass spectrometry has also been attempted in several laboratories (Millington et al. 1990; Tuchman et al. 1991; Rashed et al. 1995). Schoemaker and Elliot (1991) developed a comprehensive analytical method by urease treatment for not only organic acids, but also amino acids and carbohydrates involving GC/MS, and later Matsumoto and Kuhara (1996) developed extensively a more simplified preparation using urease with only a small amount of urine. If GC/MS is applied to screening, it will be necessary for a number of technicians or researchers to be familiar with GC/MS, the data interpretation, and pathophysiology of inherited metabolic diseases. Our system simplifies GC/MS data processing and facilitates GC/MS analysis for routine laboratory tests.

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