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Review

Diagnosis of inborn errors of metabolism using filter paper urine, urease treatment, isotope dilution and gas chromatography–mass spectrometry

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Abstract

This review will be concerned primarily with a practical yet comprehensive diagnostic procedure for the diagnosis or even mass screening of a variety of metabolic disorders. This rapid, highly sensitive procedure offers possibilities for clinical chemistry laboratories to extend their diagnostic capacity to new areas of metabolic disorders. The diagnostic procedure consists of the use of urine or filter paper urine, preincubation of urine with urease, stable isotope dilution, and gas chromatography–mass spectrometry. Sample preparation from urine or filter paper urine, creatinine determination, stable isotope-labeled compounds used, and GC–MS measurement conditions are described. Not only organic acids or polar ones but also amino acids, sugars, polyols, purines, pyrimidines and other compounds are simultaneously analyzed and quantified. In this review, a pilot study for screening of 22 target diseases in newborns we are conducting in Japan is described. A neonate with presymptomatic propionic acidemia was detected among 10,000 neonates in the pilot study. The metabolic profiles of patients with ornithine carbamoyl transferase deficiency, fructose-1,6-bisphosphatase deficiency or succinic semialdehyde dehydrogenase deficiency obtained by this method are presented as examples. They were compared to those obtained by the conventional solvent extraction methods or by the tandem mass spectrometric method currently done with dried filter blood spots. The highly sensitive, specific and comprehensive features of our procedure are also demonstrated by its use in establishing the chemical diagnosis of pyrimidine degradation defects in order to prevent side effects of pyrimidine analogs such as 5-fluorouracil, and the differential diagnosis of three types of homocystinuria, orotic aciduria, uraciluria and other urea cycle disorders. Evaluation of the effects of liver transplantation or nutritional conditions such as folate deficiency in patients with inborn errors of metabolism is also described. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Inborn errors of metabolism; Urease treatment

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1. Introduction

Since the discovery of isovaleric acidemia in 1966 [1], many inborn errors of metabolism (IEM) classified as organic acidemias, in which organic acids accumulate in urine, have been discovered using gas chromatography–mass spectrometry (GC–MS). Human urine gives evidence of the metabolism in the body and contains numerous organic acids and other chemical groups of metabolites at a variety of concentrations. GC–MS is indispensable for both qualitative and quantitative analyses of urinary metabolites, termed “metabolic profiling”. In the urine of a patient with a deficiency of an enzyme or its cofactor, the substrate of the enzyme reaction and/or the metabolites formed secondarily via by-paths due to the accumulation of the substrate, markedly increase. In some cases, instead of the substrate or the secondary metabolites, a precursor of the substrate increases due to the de-repression of end-product inhibition. Chemical diagnoses have been made by comparing the urinary organic acid profiles of patients suspected of having organic acidemias with those of controls [2,3]. Many patients with organic acidemias have been identified by such metabolic profiling [4].

Urinary organic acids are extracted with ethyl ether and/or ethyl acetate under acidic conditions with or without adding sodium chloride, and are then dehydrated with sodium sulfate and evaporated to dryness, and the residues are derivatized to increase their volatility and therefore their suitability for GC–MS analyses [2,3]. Trimethylsilylation has been used by most laboratories for this purpose. Polar organic acids such as orotate, methylcitrate and glycerol-3-phosphate are poorly recovered, and quantitative

analyses of these compounds without the respective stable isotope-labeled internal standards are difficult when using the solvent extraction method. Extraction with DEAE–Sephadex improves the recovery of polar organic acids [5], but inorganic acids such as phosphate are also well recovered thereby, which is undesirable. Furthermore, the DEAE–Sephadex procedure takes several hours.

Shoemaker et al. (1991), reported that urinary organic acids, amino acids and sugars can be analyzed simultaneously after excessive urea in urine is degraded with urease and removed [6]. Shoemaker’s procedure, however, takes several hours, needs skilled technicians, and is not very practical for the purpose of multiple sample analysis. We drastically modified and simplified Shoemaker’s procedures [7,8], based on our experiences with chemical diagnosis of IEM using GC–MS during more than two decades [9–18], and devised a procedure for multiple sample analysis and for potential use in neonatal screening. Our procedure takes 1 h for pretreatment of one sample or 3 h for a batch of 30 samples, plus 15 min (at intervals of 30 min) per sample for GC–MS measurement. Human urine contains numerous organic compounds at a variety of concentrations and gives evidence of the metabolism in the body. Our attempts to extract as much metabolic information as possible without fractionation into the organic acid fraction, amino acid fraction, polyol fraction, etc., and to use our procedure for mass screening have also been aided by the remarkable advances in GC–MS instrumentation and computer software during the last two decades.

This review will be concerned with our simple yet highly specific procedure for the diagnosis or even mass screening of a variety of metabolic disorders.

This diagnostic procedure consists of the use of urine or filter paper urine, urease-pretreatment, stable isotope dilution and GC–MS. The method, we believe, offers possibilities for clinical chemistry laboratories to extend their diagnostic capacity to new areas of metabolic disorders.

2. Experimental

2.1. Chemicals

Methylcitrate and [$^2\text{H}_3$]methylcitrate were synthesized at Cambridge Isotope Laboratory (Andover, MA, USA). [$^2\text{H}_3$]Creatinine, [$^{15}\text{N}_2$]uracil and [$^2\text{H}_3$]methionine were purchased from Isotec (Miamisburg, OH, USA), [$^{15}\text{N}_2$]orotate, [$^2\text{H}_8$]homocystine, [$^2\text{H}_4$]cystine, [$^2\text{H}_5$]glycine, [$^2\text{H}_3$]leucine, [$^2\text{H}_4$]lysine, [$^2\text{H}_5$]phenylalanine and [$^2\text{H}_4$]tyrosine from Cambridge Isotope Laboratory (Andover, MA, USA), and [$^2\text{H}_3$]methylmalonate from MSD Isotopes (Pointe-Claire-Dorval, Quebec, Canada). Urease type C-3, thymine, 5,6-dihydrothymine, 5,6-dihydrouracil and 5-fluorouracil were obtained from Sigma (St Louis, MO, USA), orotate and creatinine from Tokyo Kasei Kogyo (Tokyo, Japan) and uracil from Wako Pure Chemical Industries (Tokyo, Japan). Other reagents were from commercial sources. The purity of stable isotope-containing compounds used as internal standards was higher than 99%, except for uracil (98%), as judged by the lack of additional peaks on GC–MS. The isotope enrichment of the stable isotopes was also more than 99%.

2.2. Filter paper urine and sample preparation

Sample preparation of liquid urine samples or filter paper urine was based on the method of Matsumoto and Kuhara, which has been previously described [7,8]. Urine was poured onto a 3 cm×8 cm piece of absorbant filter paper (UA-5 from Toyo Roshi, Tokyo, Japan), which was dried in room air and sent to our laboratory for chemical diagnosis or screening of IEM. The dried filter paper urine was placed in a disposable tube. The soluble urine components were eluted with 1 ml of distilled water.

A volume of 0.7 ml of eluate was recovered and then processed as described below for liquid urine. This method of sample preparation is different from the conventional solvent extraction. It includes no fractionation, and requires urease pretreatment. A volume of 100 μl of urine is used, but, depending on the concentration of creatinine, urine volumes of 10 to 50 μl are often preferred and volumes of 200 μl are very rarely needed. Thus the size of an absorbent filter paper and the scale of the following elution can be reduced to half. The urine is incubated with 30 units of urease at 37°C for 10 min to decompose and remove excess urea present in the urine. For accurate quantification, the urine is spiked with a fixed amount of each stable isotope-labeled compound as an internal standard: 100 nmol for creatinine, 4 nmol each for uracil, orotate, and methylmalonate, 5 nmol for methylcitrate, 10 nmol each for methionine, homocystine, leucine, phenylalanine, tyrosine and cystine, 50 nmol each for glycine and lysine. Five nanomoles each of 2,2-dimethylsuccinate and 2-hydroxyundecanoate, as internal standards, and heptadecanoate, as an external standard, are also added. After deproteinization by addition of 0.9 ml of ethanol, centrifugation to remove any precipitate, and evaporation to dryness, the residue is trimethylsilylated by adding 100 μl of a mixture of bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS; 10:1, v/v) and heating at 80°C for 30 min, as described previously [7,8].

2.3. Creatinine determination

With the above method of sample preparation, creatinine in urine is quantitatively recovered. However, creatine is almost completely converted to creatinine during the procedure, as previously described by Shoemaker et al., who therefore used d_3 -creatine as an internal standard [6]. By using either d_3 -creatine or d_3 -creatinine as an internal standard, we also confirmed that the value of endogenous creatinine plus creatine is obtained [7,8]. We separately determined creatinine and creatine enzymatically by using an auto-analyzer, Beckman CX5, and evaluated the urinary metabolite levels obtained by both methods, enzymatic and GC–MS (or total).

The evaluation of metabolite levels relative to total

creatinine in urine has been reported to be especially useful during clinical episodes of patients with metabolic disorders [19]. Trimethylsilylation of creatinine produces its tri-TMS derivative (major) and di-TMS derivative (minor), and the ratio of the two is not constant. Therefore, we used d_3 -creatinine as an internal standard to quantify endogenous creatine plus creatinine but did not use it as an internal standard directly to quantify all the metabolites, as Shoemaker did [7,8].

2.4. GC–MS measurement

Aliquots (0.5 or 1 μ l) of derivatized extracts are injected into a bench-top GC–MS apparatus using an automatic injection mode with a split ratio of 1:30 (1:10–1:50).

A Hewlett-Packard GC–MSD (HP6890/MSD5973) and a Shimadzu QP5000 GC–MS are used for GC–MS measurement. Separation is carried out using a fused-silica DB-5 capillary column (30 m \times 0.25 mm I.D. with a 0.25 μ m film thickness, J&W, Folsom, CA, USA). The oven temperature is programmed to increase at the rate of 17°C/min from 60°C to 320°C, with a final holding for 10 min. The temperatures of the injection port and the transfer-line are 250°C and 300°C, respectively, and a single tapered deactivated liner is used. Electron impact mass spectra are obtained by repetitive scanning at the scan rate of 2.5 cycles/s from m/z 50 to m/z 650. After the set of analyses, the column oven temperature is kept at 290°C for 1–2 h to clean the column. Helium gas is used as carrier at a flow-rate of 1.2 ml/min. All other conditions for GC–MS measurement are the same as described previously [7,8].

3. Pilot study for newborn screening of 22 target diseases

IEM, most of which cause severe pathological consequences such as mental retardation, sudden infantile death or other irreversible conditions, can be diagnosed based on the detection in blood, urine or other physiological fluids of abnormally increased levels of compounds associated with each disorder. In many of these disorders, pathological conse-

quences can be prevented or significantly reduced by early treatment. Delayed treatment drastically reduces therapeutic effects. Therefore, early diagnosis is critical for achieving timely treatment [20]. Hence, practical, sufficiently specific, and cost-effective neonatal screening programs using filter paper blood spots, termed the Guthrie test [21], are currently conducted for six IEM in Japan. Since the 1970s GC–MS techniques have become indispensable for the chemical diagnosis of IEM, especially for organic acidurias, for high-risk individuals by selected institutions in various countries, and have been proven to be the most efficient method for their diagnosis [3,4]. GC–MS is also used for the secondary screening or scrutiny of positive cases detected by current neonatal mass screening with the Guthrie test. As for the diagnosis or for low-risk screening of large populations, such as in mass screening, however, only a limited number of projects have used GC–MS. Chamberlin et al. [22] reported in 1987 that urine filter paper was generally more useful than blood spot filter paper, except for diseases where very hydrophobic compounds accumulate. This is the reason why most laboratories throughout the world using GC–MS techniques have used and still use urine or filter paper urine rather than blood, filter paper blood spots or serum. We also confirmed this by comparing the results of GC–MS analysis using filter paper urine with those using urine, serum or filter paper blood. Tuchman et al. adopted GC–MS for a mass screening program for neuroblastoma at 3 weeks of age in Quebec, with organic solvent extraction of filter paper urine under acidic conditions [23]. This program has been further extended to the screening of acidic markers for 20 or more different metabolic conditions [24].

Dr I. Matsumoto, the pioneer in this field, has established chemical diagnostic procedures using GC–MS techniques in Japan and has conducted chemical diagnosis for high-risk patients at the request of outstanding medical institutions in Japan and even institutions in foreign countries [11,25–27]. The Japanese Society for Biomedical Mass Spectrometry was established more than 25 years ago (1975) in order to stimulate the clinical application of mass spectrometry in Japan. We have made successful chemical diagnosis of most cases with organic acidemias, ornithine transcarbamylase de-

iciency or other urea cycle disorders detected in Japan during the past two decades. Based on these experiences, a joint pilot study of neonatal screening using a simplified procedure with urease-pretreatment and GC–MS techniques was initiated in 1995 in Japan [7,8]. Specialists from four institutions (Kanazawa Medical University, Kurume University Medical School, Shimane Medical University, and Chiba Prefecture Children's Hospital) gathered and were introduced to this simplified procedure in Kanazawa Medical University in January 1995.

For the present pilot study, we examined the urine specimens taken from neonates with the informed consent of parents on day 5–7, when blood was also taken for the Guthrie test. The initially targeted 22 IEM are listed in Table 1. These diseases were targeted for reasons such as severity of the illness and effectiveness of early treatment. Neuroblastoma, which is not an IEM, was also examined although there are still opposing opinions about whether this is appropriate.

An early report about this pilot study has been

published [28]. Through the end of October 2000, 40 000 newborns were examined at the four institutions. Kanazawa Medical University screened 11 045 newborns, and detected seven cases of metabolic disorders: propionic acidemia (1 case), methylmalonic aciduria (2 case), Hartnup disease (1 case), α -aminoadipic aciduria/ α -ketoacidic aciduria (2 case) and cystinuria (1 case). The incidence of IEM in Kanazawa Medical University was thus one per 1578. Kurume University (Prof. I. Yoshida) analyzed 22 867 samples and detected 9 cases: citrullinemia (1 case), ornithine transcarbamylase deficiency (1 case), methylmalonic aciduria (1 case), propionic acidemia (2 cases), glyceroluria (2 cases), α -aminoadipic aciduria/ α -ketoacidic aciduria (2 cases), cystinuria (2 cases) and neuroblastoma (2 cases). The estimated incidence was thus one per 2540. Shimane Medical University (Prof. S. Yamaguchi) examined 4275 samples and found one case of mild phenylketonuria. Chiba Children's Hospital (Dr. M. Takayanagi) analyzed 2096 samples and did not encounter any abnormal cases. Of 40 283 newborns examined, 17

Table 1
Twenty-two target diseases

1	Methylmalonic aciduria
2	Propionic acidemia
3	Isovaleric acidemia
4	β -methylcrotonylglycinuria
5	β -hydroxy- β -methylglutaric aciduria
6	β -ketothiolase deficiency
7	Multiple carboxylase deficiency
8	Glutaric aciduria type I
9	Tyrosinemia
10	Urea cycle disorders and other hyperammonemia ^a
11	Dicarboxylic aciduria including glutaric aciduria type II
12	Lactic aciduria including fructose-1,6-bisphosphatase deficiency
13	Glyceroluria
14	Alkaptonuria
15	Pyroglutamic aciduria
16	α -Aminoadipic/ α -ketoacidic aciduria
17	Dibasic amino aciduria (cystinuria, lysinuria)
18	Nonketotic hyperglycemia
19	Maple syrup urine disease
20	Phenylketonuria·hyperphenylalaninemia
21	Galactosemia
22	Hypermethioninemia·homocystinuria
23	Neuroblastoma ^b

^a Four urea cycle disorders, ornithine transcarbamylase deficiency, citrullinemia, arginase deficiency and argininosuccinic aciduria, can be screened; for their differential diagnosis, the further analysis of arginine, argininosuccinate and citrulline is required by some other method. Deficiencies of carbamoylphosphate synthase and *N*-acetylglutamate synthase can not be screened.

^b Not an inborn error of metabolism, but easy to detect.

cases were discovered; the incidence was one per 2370 at the four institutions. As two cases of neuroblastoma were detected at Kurume University, 19 cases of disease were found (1/2120).

A method to analyze amino acids and acylcarnitines in blood spots on filter paper by tandem mass spectrometry has been developed by Millington et al. [29], Chace et al. [30] and Rashed et al. [31]. The method has recently been used for neonatal screening and has been shown to be efficient with a reasonable cost [32]. An incidence of one per 4300 babies was obtained in a pilot study which targeted amino acids and acylcarnitines in filter paper blood spots by tandem mass spectrometry conducted in Pennsylvania and North Carolina [32]. Although this method permits high-speed analyses, it might be more appropriate to refer it as a tool for screening rather than for chemical diagnosis. Therefore, the total time required to make a diagnosis and start appropriate treatment for neonates may be shorter with our method. This is because our method, in most cases, gives enough information required for a conclusive diagnosis.

Our new procedure is a highly comprehensive diagnostic tool for a wide range of metabolic disorders. For screening or making a chemical diagnosis of 22 target diseases, more than 100 compounds are quantified in this method, which allows clear chemical differentiation in a single analysis between, for instance, methylmalonic aciduria and propionic acidemia, both of which are among the most frequently observed IEM. Methylmalonic aciduria is caused by abnormally reduced activity of methylmalonyl-CoA mutase either due to a mutase apoenzyme deficiency or cobalamine synthesis defect. In blood analysis by tandem MS, propionylcarnitine is the target for both methylmalonic aciduria and propionic acidemia. In our urine analysis using GC-MS, methylmalonate is the target for methylmalonic acidemia, and methylcitrate is the target for propionic acidemia, as both methylmalonate and methylcitrate are highly cleared in the kidney. The incidence of IEM obtained by Prof. Shigematsu, Fukui Medical University in Japan, by blood/tandem mass spectrometry, was one per 20 000 neonates, and four cases of propionic acidemia were detected among 80 000 babies (personal communication).

In mass screening, many specimens must be

treated, and the quality of analysis should be consistently high. Therefore, a fully automatic pretreatment system and GC-MS conditioning are desirable. For sample preparation, we have now almost completed development of automated sample preparation using a Hewlett-Packard HP7686 Prep-Station; however, as the system is mechanically linear and there is no effective evaporation, this procedure still takes too much time.

Very recently, a GC-MS/MS method for screening urine specimens for 10 organic acidurias was described [33]; in this method, 14 markers were quantified after solid-phase extraction, oximation/trimethylsilylation (90 min for derivatization) and a very rapid GC-MS/MS measurement (10 min). As those authors used a solid-phase extraction method, analytes were restricted to organic acids. Furthermore, polar organic acids, such as methylcitrate, the most reliable index for propionic acidemia, were not targeted.

4. Propionic acidemia

Propionic acidemia (PCCD, McKusick 232000, 232050), originally described as ketotic hyperglycinemia [34], is an autosomal recessive IEM in which the activity of biotin-dependent propionyl-CoA carboxylase (propionyl-CoA: carbon dioxide ligase; PCC, EC 6.4.1.3) is deficient or greatly reduced. Propionyl-CoA is normally metabolized to give D-methylmalonyl-CoA by PCC, and the latter is nonenzymatically racemized to L-methylmalonyl-CoA, which is then converted to succinyl-CoA by vitamin B₁₂-dependent L-methylmalonyl-CoA mutase. The deficiency of L-methylmalonyl-CoA mutase either due to mutase apoenzyme deficiency or vitamin B₁₂ coenzyme deficiency causes methylmalonic acidemia (MMCMD). In patients with PCCD, propionyl-CoA, a catabolic intermediate of isoleucine, valine, methionine and threonine, undergoes altered metabolism to give methylcitrate [35], 3-hydroxypropionate, propionylglycine and other products [36]. As of 1994, we had made successful chemical diagnoses of more than 40 cases of PCCD by conventional solvent extraction and GC-MS. The urinary metabolic profiles of PCCD patients vary

significantly depending on the sampling time [12,14,37–40] as do those of methylmalonic acidemia patients [10]. In the 1970s, with our GC–MS conditions using an OV-17 liquid-phase-packed column, methylcitrate was often undetectable in the urine of PCCD patients during ketotic episodes, but other metabolites associated with PCCD increased, which enabled us to make a correct diagnosis. At present, using either solvent extraction or the simplified urease procedure, an almost conclusive chemical diagnosis can be made from a single urine specimen or filter paper urine sample. In the latter procedure, which enables high recovery and reproducibility of quantification of polar compounds such as methylcitrate and propionylglycine, simultaneous

analysis of 3-hydroxyisovalerate, 3-methylcrotonylglycine, glycine and creatinine is possible. Therefore, differential diagnosis between PCCD (Fig. 1) and multiple carboxylase deficiency, which is caused by holocarboxylase synthetase deficiency, biotinidase deficiency or biotin deficiency and results in the simultaneous deficiency of four human carboxylases (Fig. 2), or isolated methylcrotonyl–CoA carboxylase deficiency (3-methylcrotonyl–CoA: carbon dioxide ligase; EC 6.4.1.4) can be done simultaneously (Fig. 3).

Treatment for PCCD is primarily based on dietary protein restriction limiting propionyl–CoA precursors while allowing for protein anabolism by supplementation of a combination of natural milk and

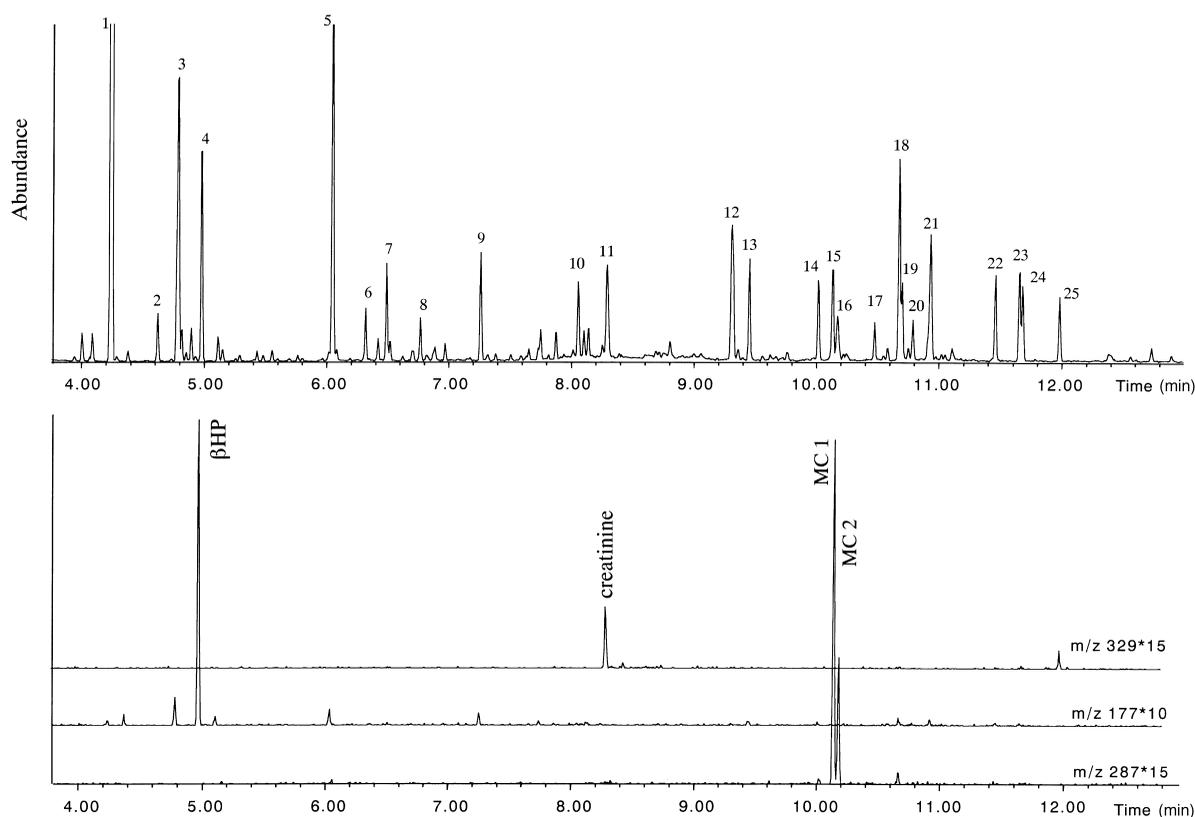


Fig. 1. Total ion current (TIC) and mass chromatograms of trimethylsilyl derivatives of metabolites from the urine of a patient with propionic acidemia during severe metabolic crisis on the 7th day of life. The ions targeted were m/z 329 for creatinine, m/z 287 for methylcitrate (MC1 and MC2) and m/z 177 for 3-hydroxypropionate (β HP). Peak identifications are: 1. lactate-2; 2. alanine-2; 3. glycine-2; 4. 3-hydroxypropionate-2; 5. phosphate-3; 6. proline-2; 7. 2, 2-dimethylsuccinate-2 (IS_1); 8. serine-3; 9. propionylglycine-2; 10. 4-hydroxyproline-3; 11. creatinine-3; 12. lysine-3; 13. 2-hydroxyundecanoate-2 (IS_2); 14. citrate-4; 15. methylcitrate-4 (1); 16. methylcitrate-4 (2); 17. glucose-5 (1); 18. galactitol-6; 19. glucitol-6; 20. tyrosine-3 and d_4 -tyrosine-3 (IS); 21. glucose-5 (2); 22. myo-inositol-5; 23. myo-inositol-6; 24. urate-4; 25. *n*-heptadecanoate-1 (IS_3).

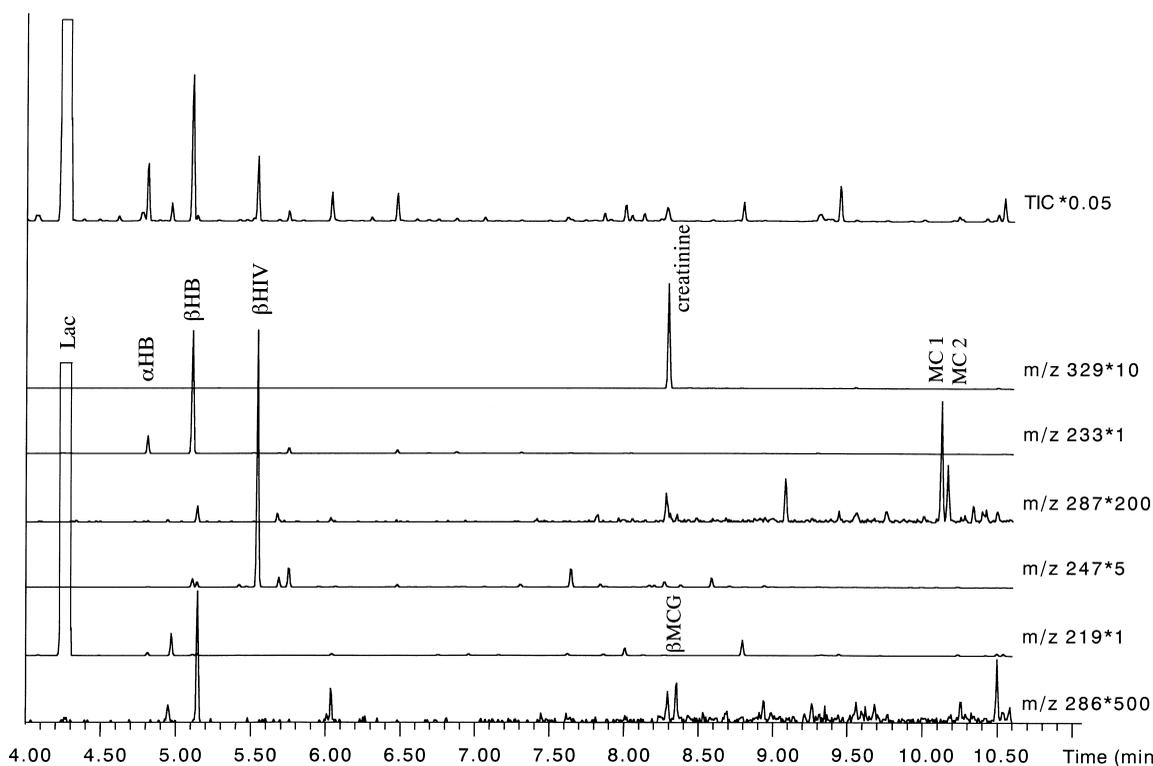


Fig. 2. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from the urine of a patient with multiple carboxylase deficiency due to holocarboxylase synthetase deficiency. The ions targeted were m/z 219 for lactate(Lac), m/z 247 for 3-hydroxyisovalerate (β HIV), m/z 286 for 3-methylcrotonylglycine di-TMS (β MCG), m/z 287 for diastereomers of methylcitrate (MC1 and MC2) and m/z 233 for 2-hydroxybutyrate (α HB) and 3-hydroxybutyrate (β HB).

artificial milk that is free from nutritionally essential amino acids producing propionyl-CoA. Clinical prognosis and long-term outcome depend essentially on metabolic control. Nevertheless, recent reports suggest that the treatment of PCCD patients still poses considerable problems; the current therapy for neonatal onset PCCD improved survival and nutritional status, but it did not appear to significantly alter the cognitive outcome, even in children with optimal metabolic control [41]. Additional therapeutic advances are therefore required to improve the developmental and cognitive outcome. Neurological abnormalities occur in some patients even in the absence of metabolic acidosis. Therefore, the diagnosis of PCCD should be considered in all newborn infants with unexplained neurological deterioration even in the absence of ketosis and metabolic acidosis [42,43]. Thus, presymptomatic diagnosis, as early as possible, is critical for the quality of life of such

patients. As neurological abnormalities in newborns are often difficult to notice, neonatal screening will meet the need for diagnosis. A different therapeutic approach, liver transplantation, was first attempted for PCCD in 1992 [44] and later in 1995 [45], but such transplants did not completely correct the metabolic abnormality due to the significant contribution of PCC from extrahepatic tissues. Combined liver-kidney transplantation in MMCMD was attempted in 1998 [46]. Both liver and liver/kidney transplantation improved the lifestyle of patients and caused a marked reduction in urinary methylcitrate [45] or methylmalonate [46]. In Fig. 4, the urinary metabolic profile of the PCCD patient after liver transplantation is shown; (the methylcitrate level was 334 mmol/mol creatinine, mean+200 SD): the same patient before liver transplantation and at chemical diagnosis made at Kanazawa Medical University at 7 days of life; (the methylcitrate level was 1610 mmol/

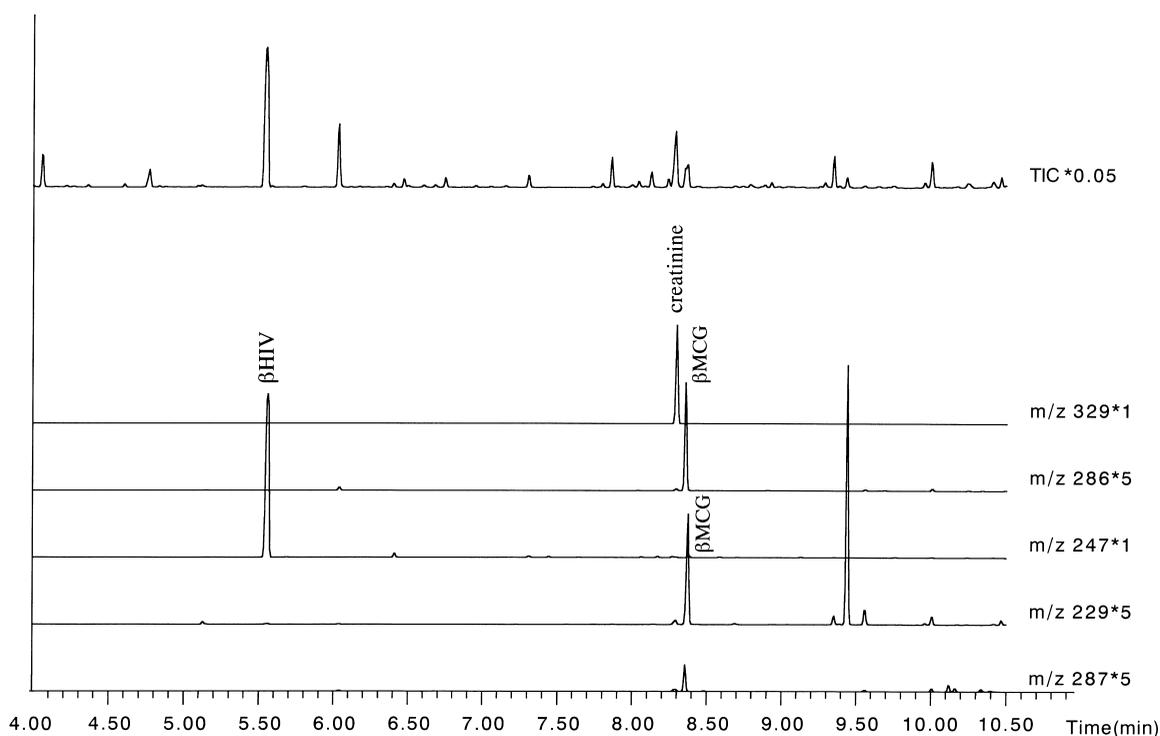


Fig. 3. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from the urine of a patient with isolated β -methylcrotonyl-CoA carboxylase deficiency. The ions targeted were m/z 329 for creatinine, m/z 286 for 3-methylcrotonylglycine (β MCG) di-TMS, m/z 229 for 3-methylcrotonylglycine (β MCG) mono-TMS, m/z 247 for 3-hydroxyisovalerate (β HIV) and m/z 287 for methylcitrate. Methylcitrate was not increased at all.

mol creatinine, see also Fig. 1). This simple yet specific procedure was shown to be one of the most useful methods for the evaluation of liver transplantation as well as for other follow-up studies.

We discovered a presymptomatic patient with PCCD in the pilot study of neonatal screening using filter paper urine, thus confirming that this simplified urease procedure is highly sensitive and specific. The methylcitrate concentration was 29 times higher than that of age-matched controls when the two diastereomers of methylcitrate were measured by routinely using 2-hydroxyundecanoate as an internal standard (Fig. 5). In Fig. 6, mass spectra of 2-hydroxyundecanoate and 2,2-dimethylsuccinate used as internal standards are shown. Methylcitrate is quantified by the stable isotope dilution method using d_3 -methylcitrate in cases in which it is significantly increased in any urine specimen. The

concentration of methylcitrate in this neonate was determined to be 35.3 mmol/mol creatinine (enzyme creatinine), 9.4 times higher than that of the control and mean+41 SD, and 18.0 mmol/mol creatinine (total creatinine obtained with GC-MS), 6.5 times higher and mean+17.7 SD. We then asked Prof. Y. Shigematsu, who is conducting a pilot study of neonatal screening by tandem mass spectrometry using blood spots in Japan, to examine the target propionylcarnithine level in blood spots of this patient taken on day 5 after birth (Guthrie blood). The level of the target was determined to be only 2.1 times higher than that of the control, mean+3.2 SD, and the ratio of propionylcarnithine to acetylcarnithine, 0.287, was slightly more marked than the cut-off point (0.21) in that method. In our procedure using urine and GC-MS, only methylcitrate was increased, but the increase of this metabolite was far more

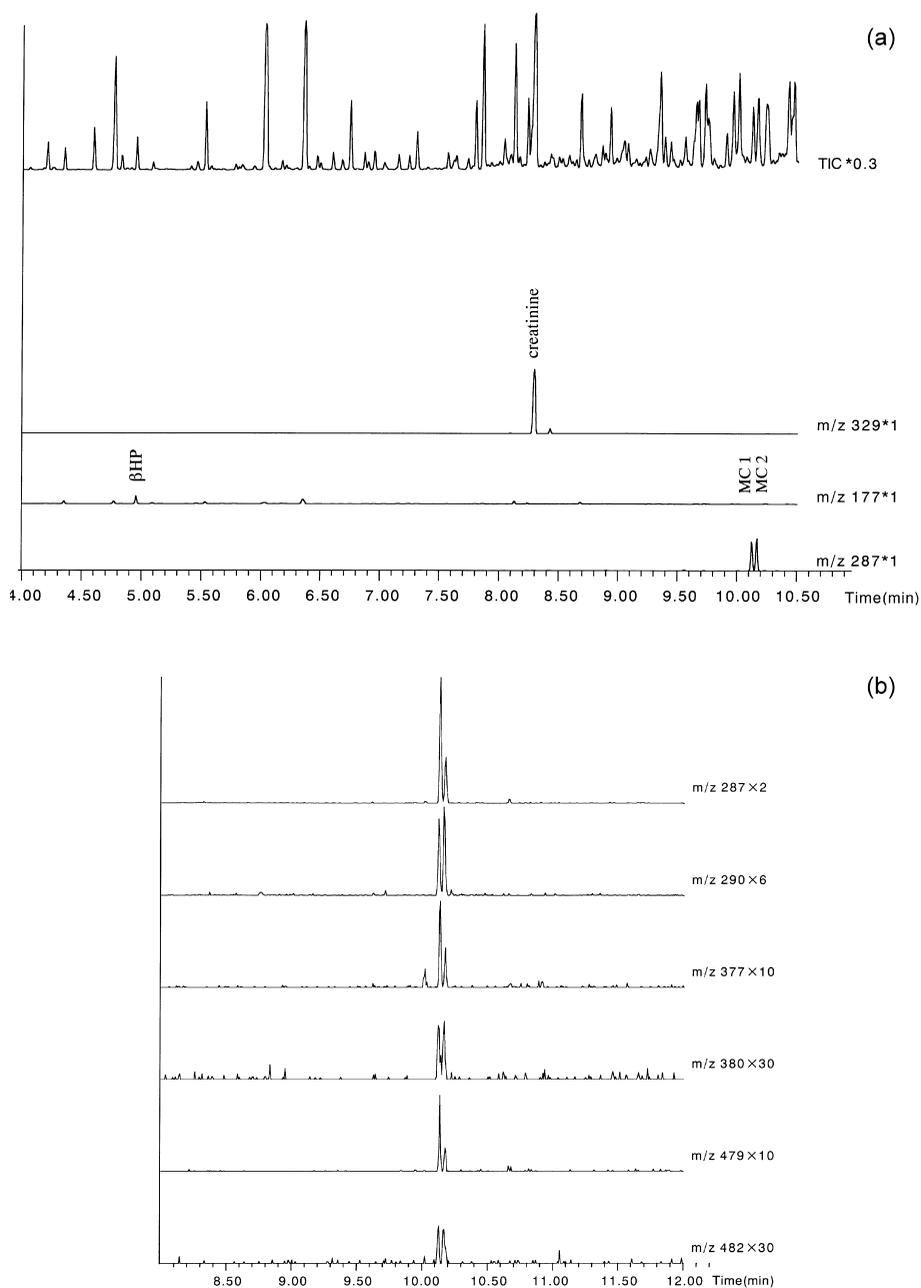


Fig. 4. (a) TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from the urine of a patient with propionic acidemia after living-related liver transplantation performed at Kyoto University [83]. The metabolic profile at chemical diagnosis made by us using the urine taken on day 7 of life is shown in Fig. 1. The ions targeted were m/z 329 for creatinine, m/z 287 for methylcitrate, MC1 and MC2, and m/z 177 for 3-hydroxypropionate (β HP). (b) Partly shown mass chromatograms of trimethylsilyl derivatives of diastereomers of methylcitrate from the urine of a patient with propionyl-CoA carboxylase deficiency (same patients as in Fig. 1). A urine specimen ($33.3 \mu\text{l}$) spiked with 10 nmol of d_3 -methylcitrate was prepared and analyzed for GC-MS. Ions at m/z 479 and 482 (M-15), m/z 377 and 380 (M-COOTMS), and m/z 287 and 290 (M-COOTMS-TMSOH) for cold and labeled methylcitrate, respectively.

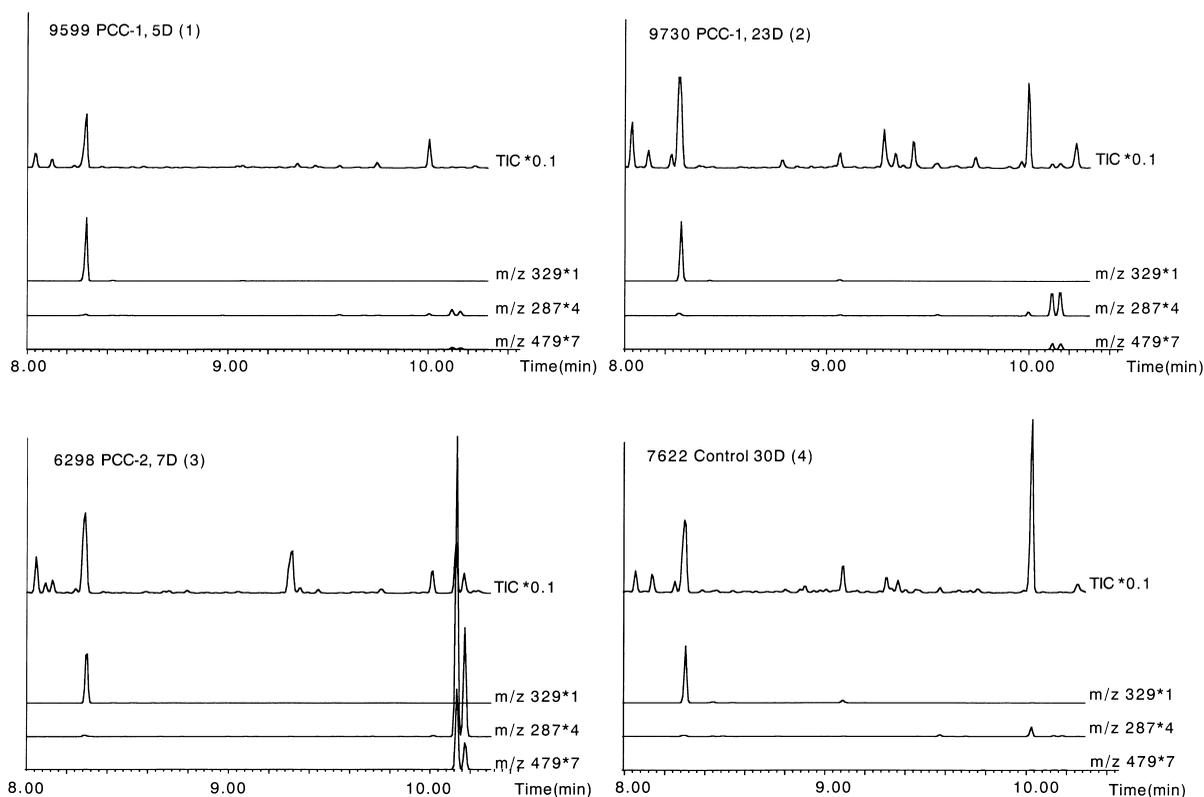


Fig. 5. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from the filter paper urine of a 5-day-old neonate in whom the chemical diagnosis of propionic acidemia was made based on this profile, during the pilot study, and later confirmed by measurement of the PCC activity by Assoc. Prof. N. Sakura, Hiroshima Univ. (1), from the same patient at 23 days of life (2), from the urine of a patient with neonatal onset propionic acidemia on day 7 after birth (3) and from a 30-day-old healthy infant as a control (4). The ions targeted were m/z 329 for creatinine, and m/z 287 and m/z 479 for methylcitrate.

marked than the increase seen in the blood tandem method, that is, the methylcitrate level in urine was 6.5 times higher than that of the control (Table 2).

Just before starting the pilot study, we compared the levels of our targets for PCCD and confirmed that methylcitrate and 3-hydroxypropionate were detected in large amounts in urine, but only the former in lower amounts in the serum of patients with PCCD [28]. In our pilot study, in neonates with benign methylmalonic aciduria, there was a marked increase of methylmalonate in the urine taken on day 5 and eluted from dried filter paper while there was no such increase in the serum. The methylmalonate concentration was increased more than 100 fold compared with that of the age-matched controls when methylmalonate was measured by routinely

using 2,2-dimethylsuccinate as an internal standard. Methylmalonate is quantified by the stable isotope dilution method in cases in which it is significantly increased in any urine specimen. The concentrations of methylmalonate in the neonates were determined to be 0.6 and 0.7 mmol/mol creatinine (enzyme creatinine), respectively. Blood spots on filter paper from the same patient did not show any abnormal signal on tandem mass spectrometry (MS/MS) conducted either by Prof. C. Roe and Prof. L. Sweetman, or by Prof. Y. Shigematsu, showing the difficulty of detecting propionylcarnitine, which is the target for MMCMD, with blood spots. This may, however, have been partly due to weakness of the block of the methylmalonate metabolism in our patients.

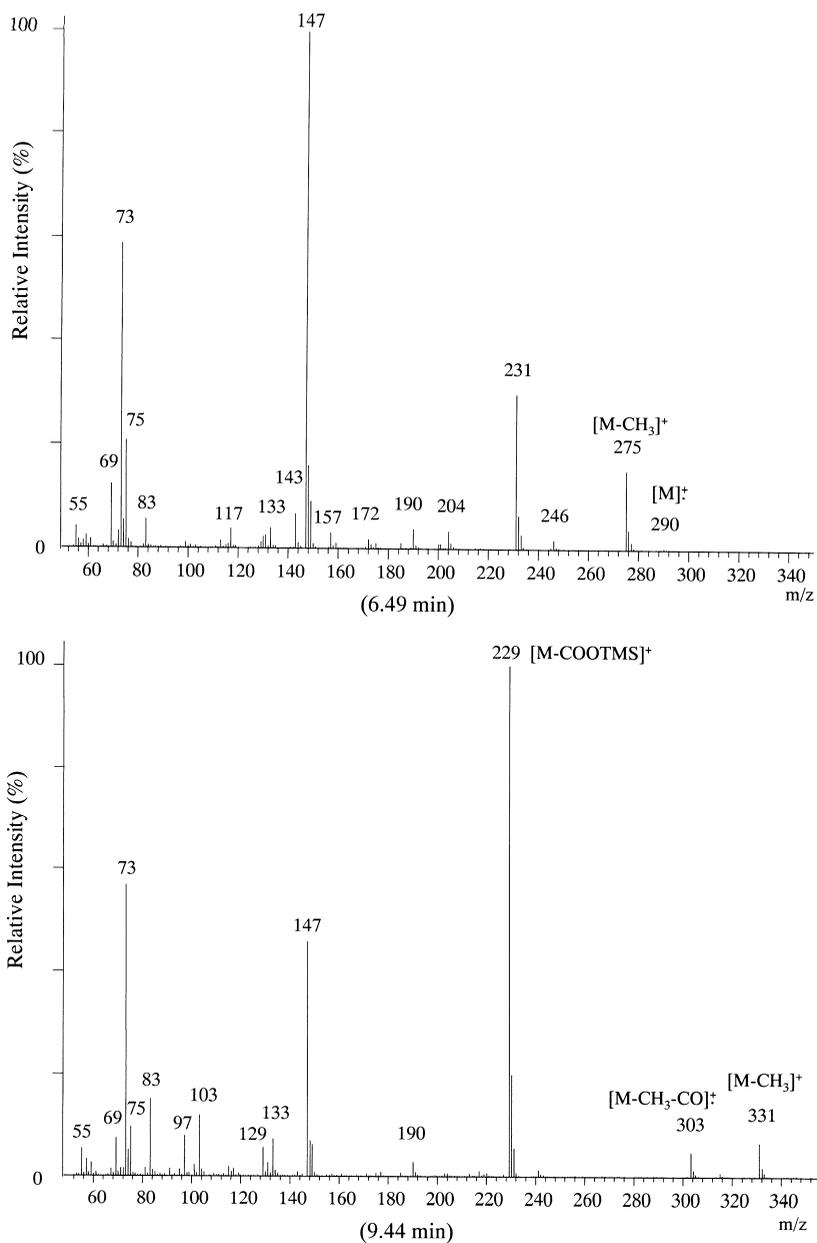


Fig. 6. Mass spectra of trimethylsilyl derivatives of two internal standards, 2,2-dimethylsuccinate (upper) and 2-hydroxyundecanoate (lower). The ion at m/z 231 of 2,2-dimethylsuccinate di-TMS and the ion at m/z 229 of 2-hydroxyundecanoate di-TMS were used to quantify the several metabolites that eluted earlier or later on the chromatogram, respectively.

5. Ornithine carbamoyltransferase deficiency and other hyperammonemia

Uracil and/or orotate are increased in the urine of patients with hyperammonemia due to the deficiency

of any enzyme involved in the urea cycle except for carbamoylphosphate synthase and *N*-acetylglutamate synthase. In these patients, carbamoylphosphate accumulates in mitochondria. This elevated level of carbamoylphosphate of mitochondrial origin causes

Table 2
Comparison of abnormality magnitude of target in two methods

Method	Target	Magnitude ^a <i>n</i> in <i>M</i> + <i>n</i> SD	Fold
Filter paper urine GC/MS (present procedure)	Methylcitrate	17.7 (41)	6.5(9.4)
Filter paper blood MS/MS	Propionylcarnitine ^b	3.2	2.1

^a Compared with healthy newborns.

^b Determined by Prof. Y. Shigematsu, Fukui Medical College, Japan PC/AC: 0.287, cut-off <0.21 in filter paper blood·MS/MS.

the increased de novo synthesis of pyrimidine in the cytosol, resulting in the increase of orotate and/or uracil, which are therefore targeted for the screening of these disorders [47]. For patients with orotic aciduria and/or uraciluria, the levels of citrulline and arginine are determined using a conventional amino acid autoanalyzer, or soft ionization MS, such as FAB- or ESI (not GC–MS), and such patients are differentially diagnosed for ornithine carbamoyltransferase deficiency, citrullinemia, argininosuccinic aciduria, arginase deficiency or HHH syndrom. Just before starting our pilot study, we compared the levels of our targets and confirmed that uracil and orotate were detected in large amounts in urine, but only the former in lower amounts in the serum of patients with deficiency of ornithine carbamoyltransferase (carbamoylphosphate: L-ornithine carbamoyltransferase; EC 2.1.3.3) [28]. Therefore, analysis of urine is far more sensitive than analysis of serum.

Stable isotope dilution GC–MS analysis is well suited for the quantitation of orotate even in dried filter paper urine specimens, as reported [48]. The extraction efficiency of orotate obtained using stable isotope-labeled orotate as an internal standard was 31% in the reported solvent extraction method (ethyl acetate twice with sodium chloride under acidic conditions) [48], but was 83% in our simplified diagnostic procedure. This procedure, therefore, appears to be suitable also for the evaluation of the female carrier status [49]. In Fig. 7 (upper), TIC and mass chromatograms of TMS derivatives of metabolites of the same urine specimen from a patient with ornithine carbamoyltransferase deficiency obtained even after protein restriction are shown. Orotate was markedly increased. In our conventional solvent

extraction (ether twice without sodium chloride under acidic conditions), the recovery of orotate relative to that of uracil was also very poor (Fig. 7 lower), but in our simplified urease treatment, the recovery was as high as 83%. For patients with ornithine carbamoyltransferase deficiency, liver transplantation has been tried [50]. In male infants who received liver transplants in Japan, urinary uracil and orotate disappeared when determined by our procedure, and it was found that this procedure is also one of the most useful methods for the evaluation of liver transplantation for patients with urea cycle disorders.

6. Fructose-1,6-bisphosphatase deficiency

Fructose-1,6-bisphosphatase (D-fructose-1, 6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11) is a key enzyme of gluconeogenesis. Deficiency of fructose-1,6-bisphosphatase (MIM 229700), originally described in 1970 [51], therefore causes severe lactic acidemia and hypoglycemia during fasting conditions [52]. During remissions, the urinary metabolic profiles appear normal as compared with those of controls. Therefore, it becomes very difficult to make a chemical diagnosis. During a hypoglycemic episode, however, the metabolic profile changes dramatically: lactate, glycerol and glycerol-3-phosphate all markedly increase in urine [53]. Lactate and glycerol are, however, not specific markers, as the former increases under a variety of disease conditions and the latter as a result of the glycerol infusion treatment often administered. Glycerol-3-phosphate, a very polar organic acid, is poorly recovered, and quantitative analysis of its level

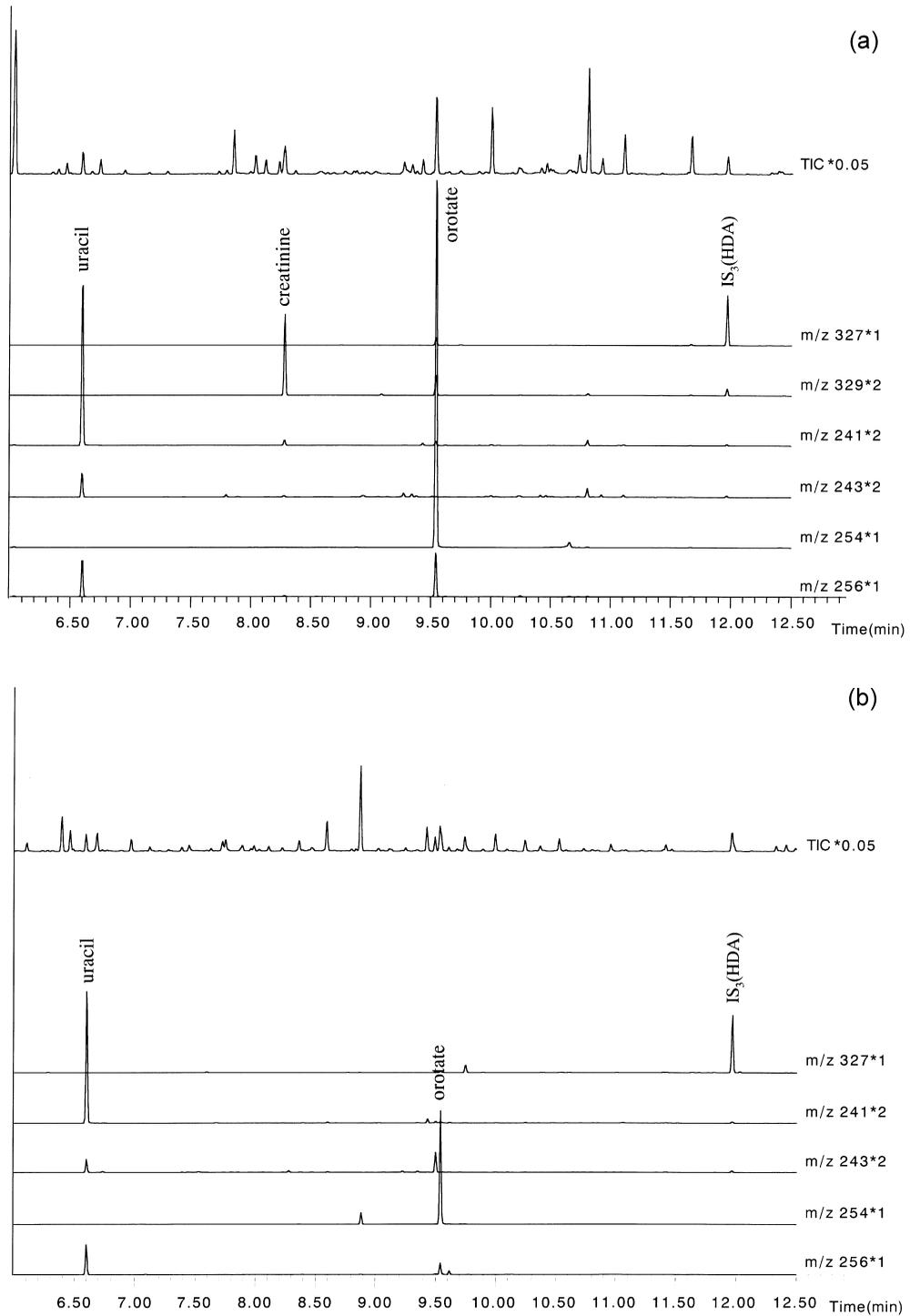


Fig. 7. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from the urine of a patient with ornithine carbamoyltransferase deficiency processed by simplified urease treatment (upper), and the solvent extraction method (lower). The ions targeted were m/z 327 for IS₃, (external standard heptadecanoate; HDA), m/z 329 for creatinine, m/z 241 and m/z 243 for uracil (cold and labeled) and m/z 254 and m/z 256 for orotate (cold and labeled).

without the respective stable isotope-labeled internal standard is difficult in the solvent extraction method. Extraction with DEAE–Sephadex significantly improves the recovery of glycerol-3-phosphate [5,54], but inorganic acids such as phosphate or sulfate are also well recovered, which is inconvenient for the subsequent GC–MS analysis, and glycerol cannot be recovered. Furthermore the DEAE–Sephadex procedure takes several hours. In our simple urease treatment procedure, recovery of this polar acid is very high, as in the DEAE–Sephadex method, and the diagnosis of this disease becomes remarkably rapid, accurate and easy, as has been reported [8]. Very recently, more sensitive quantification with the single ion monitoring (SIM) mode for glycerol-3-phosphate, glycerol and lactate with the simple urease pretreatment method was described [55], but using our routine analytical conditions, all three are adequately detectable in healthy controls, even in the scanning mode described in Experimental. Fig. 8 shows the TIC and mass chromatograms of TMS derivatives of metabolites from a patient diagnosed by us as having fructose-1,6-bisphosphatase deficiency, obtained using the present procedure during an episode (upper) and remission (lower).

7. Succinate semialdehyde dehydrogenase deficiency

Deficiency of succinate semialdehyde dehydrogenase (succinate semialdehyde: NAD^+ oxidoreductase, SSADH; EC 1.2.1.24), also known as 4-hydroxybutyric aciduria (McKusick 271980), is an autosomal recessive inborn error in the metabolism of the neurotransmitter 4-aminobutyrate. Predominant oxidative conversion of succinate semialdehyde to succinate is impaired, and consequently succinic semialdehyde is reduced to 4-hydroxybutyrate in a reaction catalyzed by 4-hydroxybutyrate dehydrogenase (EC 1.1.1.61). The first patient with this disease was described by Jacob et al. in 1981 [56]. The initial step in diagnosis is usually the recognition of excessive urinary excretion of 4-hydroxybutyrate and 3,4-dihydroxybutyrate; the latter is thought to be formed by β -oxidation [57]. Other minor metabolites have also been reported [58]. These metabolites were detected by the conventional solvent extraction pro-

cedure, in which organic acids are extracted from acidified urine using ethyl ether and ethyl acetate and converted to trimethylsilyl derivatives [58]. It is known that, during evaporation of organic solvents, lactone formation, which to some extent also occurs in vivo [58], is enhanced, resulting in apparently low levels of these hydroxy acids [59], thereby making the chemical diagnosis difficult. The TIC chromatograms of trimethylsilyl derivatives of urinary metabolites obtained by the urease treatment described in the present study from the urine of a patient who was chemically diagnosed by us as having succinic semialdehyde dehydrogenase deficiency are shown in Fig. 9a. The profile of the same urine specimen was analyzed by ether extraction was compared (Fig. 9b, upper and lower). The ratio of 3,4-dihydroxybutyrate relative to 4-hydroxybutyrate was much higher in sample with the urease treatment than in that with ether extraction, suggesting that the recovery of polar 3,4-dihydroxybutyrate is better and that this new procedure would improve the sensitivity of diagnosis of patients with this disease and lower the rate of false negatives. Recently, the clinical heterogeneity and response to early treatment with vigabatrin in siblings with succinic semialdehyde dehydrogenase deficiency was reported [60]. The author also found significant differences of the urinary metabolite levels among siblings (unpublished observations).

8. Deficiencies of pyrimidine degradation

Inborn errors of pyrimidine degradation are less rare than has generally been assumed, and clinical presentations of such patients are variable and non-specific [61–63]. Pyrimidines are degraded in four steps in humans, catalyzed by dihydropyrimidine dehydrogenase (5,6-dihydropyrimidine: NADP^+ oxidoreductase; DHPDH, EC 1.3.1.2), dihydropyrimidinase (5,6-dihydropyrimidine amidohydrolase; DHP, EC 3.5.2.2), β -ureidopropionase (UP, EC 3.5.1.6), and three aminotransferases. Many asymptomatic cases with DHPDH deficiency have been reported, and for cases with symptoms, the clinical abnormalities are variable and nonspecific. Asymptomatic infants and adults with DHP deficiency have also been reported [63,64]. No treatment specific

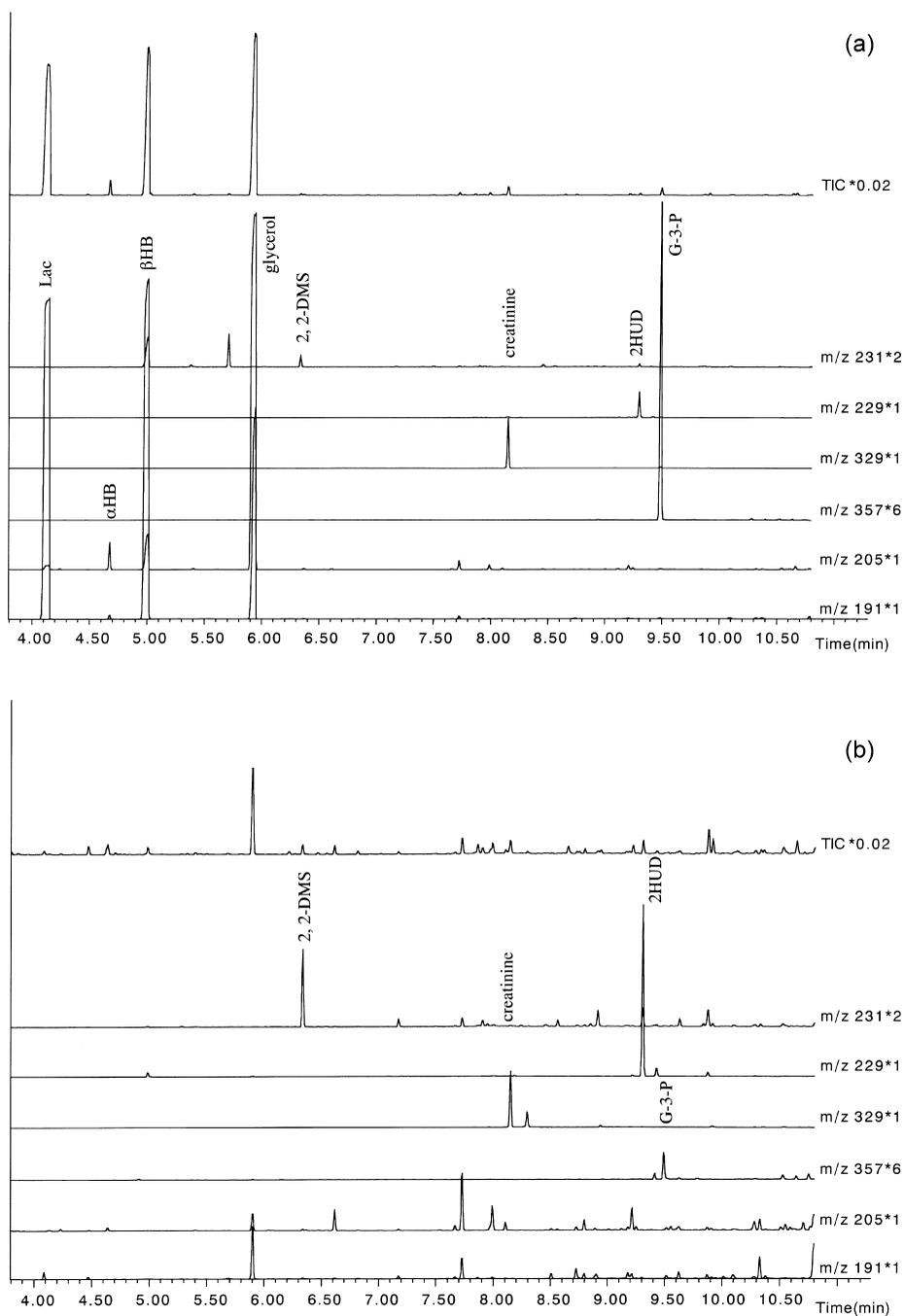


Fig. 8. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from urine of a patient with fructose-1,6-bisphosphatase deficiency. Both were obtained using the simplified urease treatment: during a hypoglycemic episode (upper), and during a remission (lower). The ions targeted were m/z 231 for 2,2-dimethylsuccinate (2,2-DMS, IS₁), m/z 229 for 2-hydroxyundecanoate (2HUD, IS₂), m/z 329 for creatinine, m/z 357 for glycerol-3-phosphate (G-3-P), m/z 205 for glycerol and m/z 191 for lactate (Lac). During the episode 3-hydroxybutyrate (β HB) is also increased. Glycerol-3-phosphate is markedly reduced but still detectable during remission of the patient and in the control even in the scanning mode.

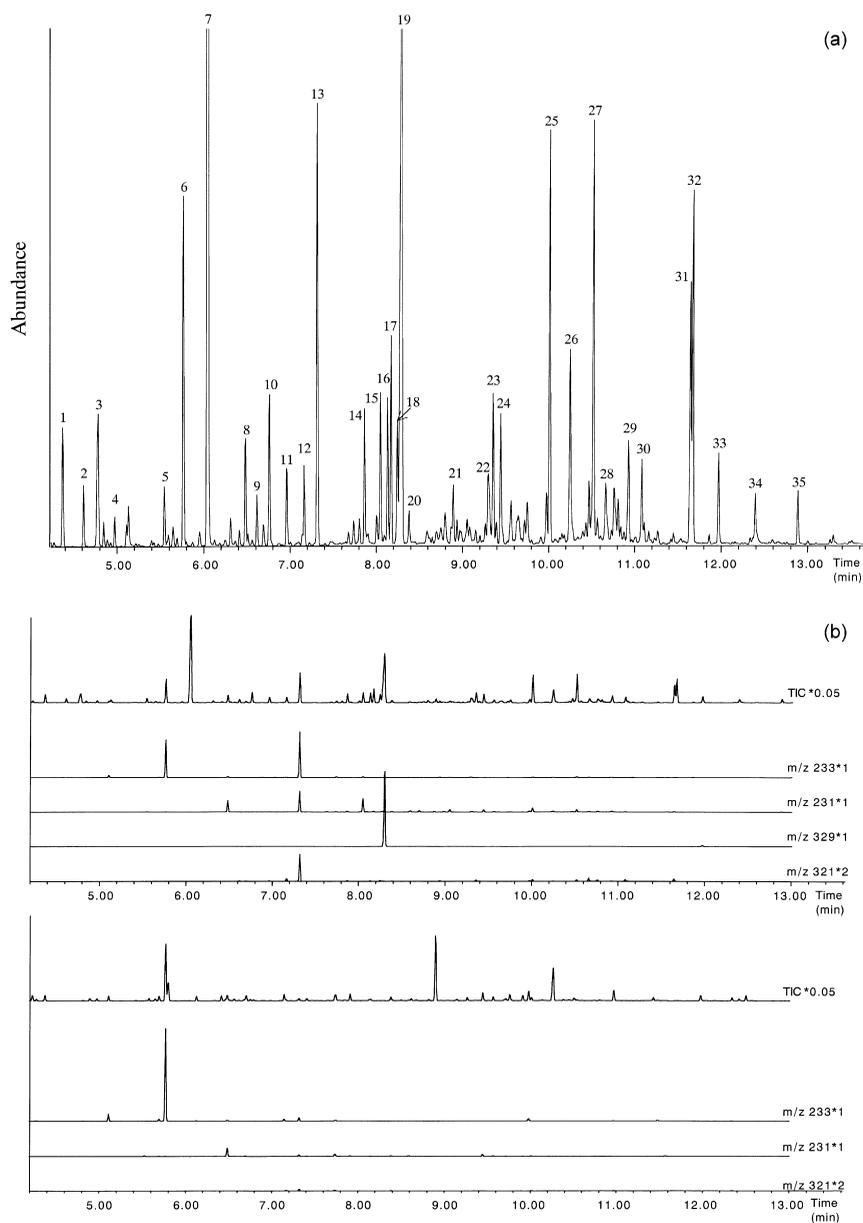


Fig. 9. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from the urine of a patient with succinic semialdehyde dehydrogenase deficiency. The present simplified urease treatment (upper and upper part of lower) and conventional solvent extraction (lower). The ions targeted were m/z 329 for creatinine, m/z 233 for 4-hydroxybutyrate, m/z 321 for 2-deoxytetronate and m/z 231 for 2,2-dimethylsuccinate added as internal standard respectively. Ions at m/z 233(M-103) and 231(M-105) are also present in 2-deoxytetronate. Peak identifications are: 1. glycolate-2; 2. alanine-2; 3. glycine-2 and d_2 -glycine (IS); 4. 3-hydroxypropionate-2; 5. 3-hydroxyisovalerate-2 and 2-aminoisobutyrate-2; 6. 4-hydroxybutyrate-2; 7. phosphate-3; 8. 2,2-dimethylsuccinate-2 (IS₁); 9. uracil-2 and $^{15}N_2$ -uracil-2 (IS); 10. serine-3; 11. threonine-3; 12. 3-deoxytetronate-3; 13. 2-deoxytetronate-3; 14. xylitol-5; 15. 4-hydroxyproline-3 and 5-oxoproline-2; 16. threonate-4; 17. threo- and erythro-4,5-dihydroxyhexanoate; 18. erythronate-4; 19. d_3 -creatinine-3 (IS) and creatinine-3; 20. 2-hydroxyglutarate-3; 21. *p*-hydroxyphenylacetate-2; 22. d_4 -lysine-3 (IS) and lysine-3; 23. 2-aminoadipate-3; 24. 2-hydroxyundecanoate-2 (IS₂); 25. citrate-4; 26. galactose-5(1); 27. galactose-5 (2); 28. histidine-3; 29. glucose-5 (2); 30. galactonate-6; 31. myo-inositol-6; 32. urate-4; 33. *n*-heptadecanoate-1 (IS₃); 34. unknown; 35. pseudouridine-5.

for these enzyme defects has been described, but withdrawal of 5-fluorouracil (5FU), a commonly used anticancer drug, from cancer chemotherapy regimens of patients with these defects is critical because 5FU is degraded *in vivo* by these enzymes. Patients with these deficiencies suffer from severe neurotoxicity, sometimes leading to death, following administration of 5FU, and even otherwise asymptomatic homozygotes or heterozygotes may develop severe clinical symptoms due to such medication. Therefore, rapid and specific identification of the cancer patients with these enzyme deficiencies is critical prior to treatment with 5FU. DHPDH deficiency is characterized by the presence of abnormal amounts of uracil and thymine in urine [65] because DHPDH is the initial and rate-limiting enzyme in the catabolism of the pyrimidine base. DHP deficiency can also be detected by the presence of large amounts of dihydrouracil and dihydrothymine, and moderate amounts of uracil and thymine, in urine [66]. Several methods to screen for disorders of pyrimidine metabolism that use HPLC [67,68], two dimensional thin-layer chromatography [69], amino acid analysis of urine before and after acid hydrolysis [70], gas chromatography, or GC–MS [71] have been reported. However, these methods have been time-consuming or have lacked specificity or sensitivity. Identification by HPLC of all the specific metabolites excreted in DHP deficiency is difficult because the maximal UV-absorbance of the dihydropyrimidines occurs below 230 nm [72]. GC–MS analysis of trimethylsilyl derivatives of urinary organic acid extracts could detect this deficiency [7] but it has been pointed out that quantitation was not possible because of variable extraction yields, and that methods involving use of two-dimensional TLC or HPLC with or without prefractionation of urine were more sensitive [62]. Very recently, a rapid and specific screening method for patients at risk of inherited disorders of pyrimidine and purine metabolism was described: it involves the use of urine and HPLC–ESI–MS/MS [73] where uracil, thymine, 5-hydroxymethyluracil and orotate were targeted, but not dihydrothymine, dihydrouracil, creatinine or amino acids.

The simplified procedure described here was applied to identify patients with these defects in the pyrimidine degradative pathway by targeting these

pyrimidines and orotate using $^{15}\text{N}_2$ -uracil and $^{15}\text{N}_2$ -orotate as their respective internal standards. We prepared artificial urine specimens which simulated typical, moderate and heterozygotes of DHPDH deficiency and those of DHP deficiency, by spiking a urine specimen with different amounts of uracil, thymine and/or dihydrouracil and dihydrothymine, and confirmed that the chemical diagnosis can be done. Recovery and CV were satisfactory, and the values of healthy controls were determined [74]. The method was established for rapid, highly sensitive and specific determinations of thymine, uracil, dihydrothymine, dihydrouracil, orotate and creatinine simultaneously in 0.1-ml liquid urine samples or filter paper urine.

9. Differential diagnosis of homocystinuria

The transsulfuration pathway converts the sulfur atom of methionine into the sulfur atom of cysteine, and reforms methionine by methylation of homocysteine. Homocystinuria types I, II and III are characterized by different etiologies, biochemical abnormalities and therapeutic measures. In type I, due to a deficiency of cystathionine β -synthase (L-serine hydrolyase (adding homocysteine); EC 4.2.1.22), homocysteine accumulated in this disorder causes methionine overproduction. A simple treatment with pyridoxine for the pyridoxine-responsive type, or a dietary restriction of methionine and supplementation with cystine for the pyridoxine-unresponsive type, greatly improve the outcome of affected infants [75]. Homocystinuria type II is caused by defective remethylation due to the deficiency of $\text{N}^{5,10}$ -methylene tetrahydrofolate reductase (5-methyltetrahydrofolate: (acceptor) oxidoreductase; EC 1.1.1.15) (MTHFR, EC 1.1.1.68). Folate and betaine may have the advantage of lowering homocysteine levels and increasing methionine levels [76]. Recently, the importance of the role of folate and of early detection of type II patients has been stressed [77,78]. Homocystinuria type III is caused by the deficiency of N^5 -methyltetrahydrofolate homocysteine methyltransferase (*S*-adenosyl-L-methionine: L-homocysteine *S*-methyltransferase; EC 2.1.1.10) due to the defective synthesis of methylcobalamin and deoxyadenosylcobalamin. This condition or nutri-

tional vitamin B₁₂ deficiency is accompanied by combined homocystinuria and methylmalonic aciduria [79]. As current neonatal screening for homocystinuria type I targets methionine in filter paper blood spots, type II is not detected, since it causes moderate homocystinuria with low or relatively normal levels of plasma methionine. Instead, isolated hypermethioninemia due to a deficiency of hepatic methionine adenosyltransferase (*S*-adenosylmethionine synthetase, ATP: L-methionine *S*-adenosyltransferase; EC 2.5.1.6) is screened as well, and it is clinically free of symptoms, indicating that the accumulation of methionine in the body is not harmful. The simplified diagnostic procedure has proven to be able to differentiate the three types of homocystinuria by simultaneous quantification of methionine, homocystine, methylmalonate, uracil and creatinine in filter paper urine, when each respective stable isotope-labeled compound is used, as reported recently [80]. The mass chromatograms of trimethylsilyl derivatives of metabolites from patients with type I and type II disease are shown in Fig. 10.

Urinary metabolite levels determined by using the simplified urease procedure were compared before and after treatment with folate in a male patient who temporarily developed megaloblastic anemia (Fig. 11). As his serum folate was below the normal range, he was treated with folate, after which megaloblastic anemia disappeared and the level of orotate decreased into the normal range. Thymidilate synthase, which catalyzes the conversion of dUMP to dTMP, is folate-dependent, and pyrimidine biosynthesis is regulated by end-product inhibition. Folate deficiency thus causes impaired DNA synthesis, enhanced pyrimidine biosynthesis, megaloblastic anemia and orotic aciduria. Folate supplementation significantly reduced the level of homocystine and dramatically increased that of methionine. This simple diagnostic procedure has therefore proved useful for monitoring the biochemical and nutritional conditions of patients, especially for acquired deficiency of folate and vitamin B₁₂, as well as for evaluating the efficacy of treatments.

The present method enables us to obtain numerous kinds of information about the metabolism in the human body. However, a variety of compounds are recovered and yet derivatized only by trimethyl-

silylation, and GC–MS conditioning thus becomes more important for continuous GC–MS measurement. It is hoped that a guard column can be successfully applied to address this problem. For amino acids, trimethylsilylation is not always quantitative. Therefore, in our procedure stable isotope-labeled internal standards are used for important amino acids and labeled omega-amino acid is used as the internal standard for omega-amino acids. In some reports this simplified urease-GC–MS method is termed the urease/direct method [81], and instead of trimethylsilylation, *tert*-butyldimethylsilylation has been recommended [82], and reported to be useful for screening for organic acidemias [81]. However, if sample preparation or GC–MS conditioning is inadequate, measurements of not only amino acids but also organic acids such as methylcitrate become less quantitative, and the sensitivity of their detection is lowered. As the *tert*-butyldimethylsilyl moiety is bulkier than the trimethylsilyl moiety, the tendency not to be fully silylated and to give several derivatives may be higher for *tert*-butyldimethylsilylation, especially for polyols and sugars. Under these conditions, data analysis may become more complicated after repeated GC–MS measurements.

We believe that the present procedure will provide valuable tool for screening of more than 80 target diseases. This procedure, technically practical yet comprehensive from the metabolic point of view, could become well established for screening of all age groups ranging from neonates to the elderly.

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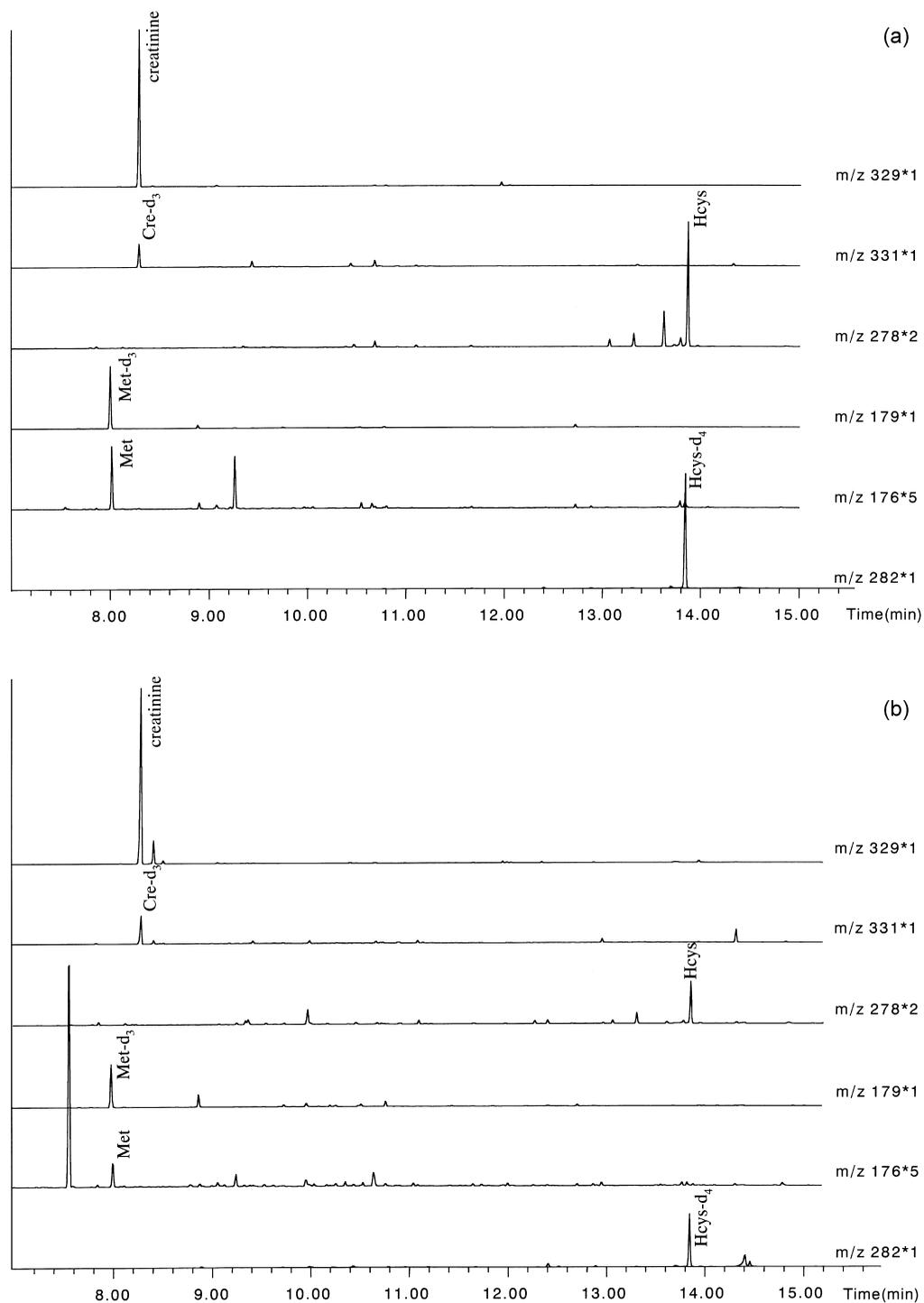


Fig. 10. Partly shown mass chromatograms of trimethylsilyl derivatives of metabolites from urine of patients with homocystinuria of type I (upper) and type II (lower). Methionine (Met) concentration in the patient with type I was significantly reduced on this occasion due to folate deficiency. The ions targeted were m/z 329 and 331, m/z 176 and 179 and m/z 278 and 282 for creatinine and d_3 -creatinine, methionine and d_3 -methionine, and homocystine and d_4 -homocystine, respectively.

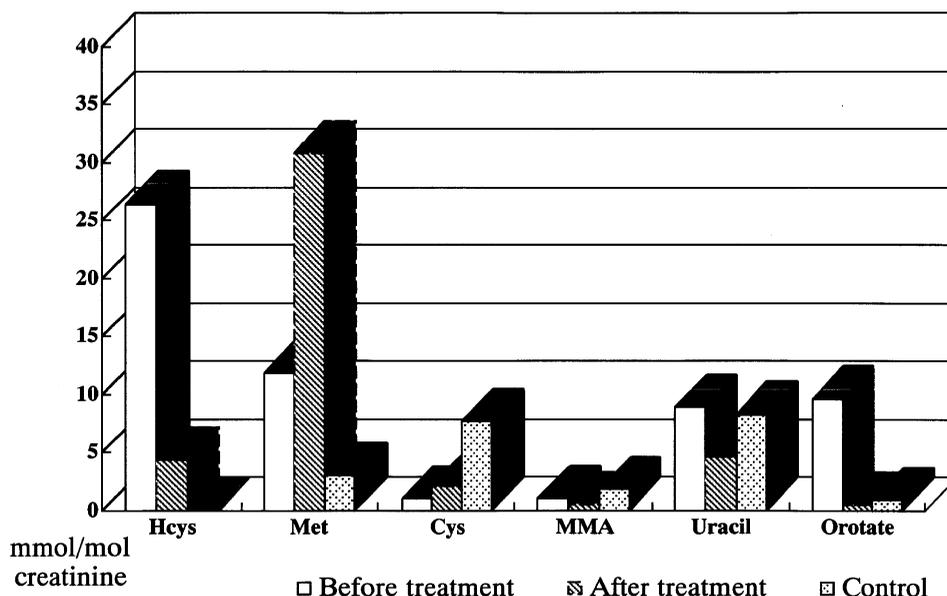


Fig. 11. Metabolite levels in homocystinuria patient before and after treatment of megaloblastic anemia by administration of folate [80].

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