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Differential diagnosis of homocystinuria by urease treatment, isotope dilution and gas chromatography–mass spectrometry☆

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Abstract

Homocystinuria types I, II and III are characterized by different etiologies, biochemical abnormalities and therapeutic measures. For this reason, differential diagnosis is critical for effective treatment. We describe here a rapid and simple procedure for establishing a differential diagnosis of the three types of homocystinuria by analyzing the urine of patients. This procedure, which consists of urease treatment, stable isotope dilution and GC–MS, enables a simultaneous quantification of methionine, homocystine, cystine, methylmalonate, orotate, uracil and creatinine. Analysis with this procedure showed that a case of homocystinuria type I, who progressed into transient megaloblastic anemia, secondarily excreted an increased concentration of orotate, which normalized after treatment with folate and vitamin B_{12}. Therefore, the present diagnostic procedure not only enables rapid differential diagnosis of homocystinuria, but also should prove useful for monitoring the disease state and understanding the nutritional condition and therapeutic state of patients, which in turn can be used to evaluate the efficacy of treatment.

Keywords: Homocystinuria; Urease; Enzymes

1. Introduction

The trans-sulfuration pathway converts the sulfur atom of methionine into the sulfur atom of cysteine and reforms methionine by methylation of homocysteine. Increased urinary excretion of homocystine is found in three types of homocystinuria (types I, II and III). Type I, clinically the most severe type, is characterized by ocular, cardiovascular, neurological, and skeletal changes, and is due to cystathionine β-synthase (EC 4.2.1.22) deficiency (Fig. 1). For the pyridoxine-responsive type, simple treatment with pyridoxine, and for the pyridoxine-unresponsive type, dietary restriction of methionine and supplementation with cystine, improves the outcome of affected infants [1]. Homocystinuria type II is characterized by defective remethylation due to $N^{5,10}$-methylenetetrahydrofolate reductase (MTHFR, EC 1.1.1.68) deficiency. The major biochemical findings of MTHFR deficiency are moderate homocystinuria with low or relatively normal levels of plasma methionine. Clinical symptoms are developmental

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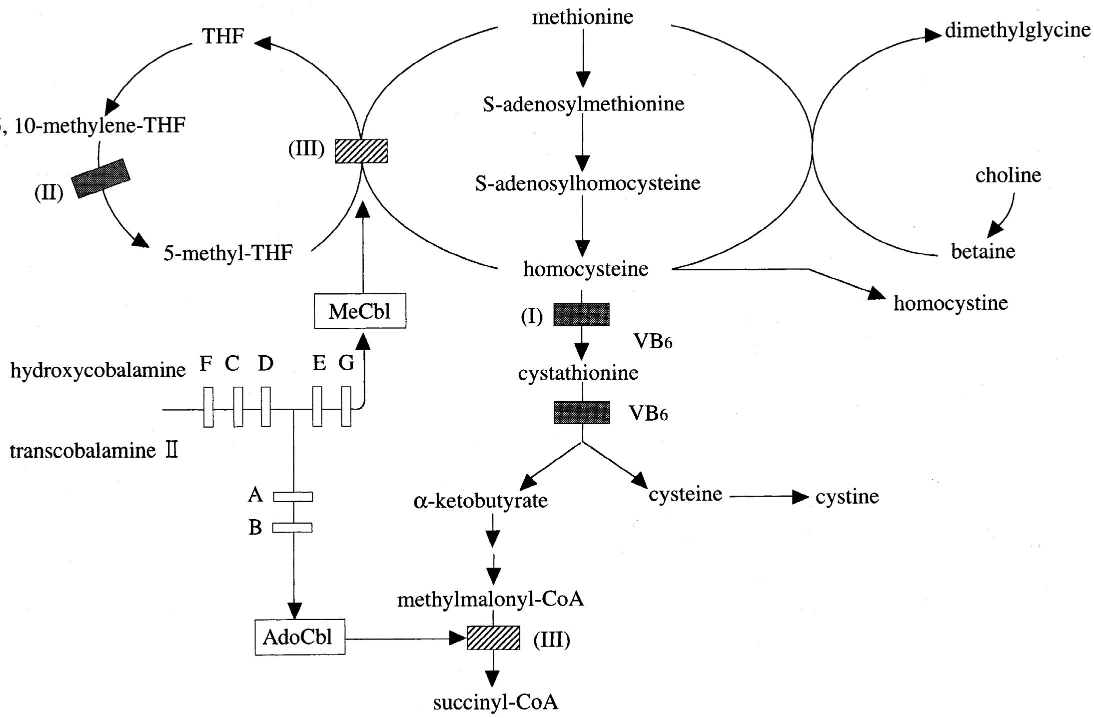


Fig. 1. Disorders of trans-sulfuration and enzyme deficiency: cystathionine-β-synthase deficiency (I), 5,10-methylene THF reductase deficiency (II), coenzyme deficiency for 5-methyl-THF, homocysteine methyltransferase and methylmalonyl-CoA mutase due to either nutritional B_{12} deficiency or B_{12} activation disorder at F, C, or D (III). THF, tetrahydrofolate.

delay accompanied by motor and gait abnormalities, seizures, and psychiatric manifestations. Folate and betaine may have the advantage of lowering homocysteine levels and supplementing methionine levels [2]. Homocystinuria type III is caused by N^5-methyltetrahydrofolate homocysteine methyltransferase deficiency due to the defective synthesis of methylcobalamin and deoxyadenosylcobalamin. This condition or nutritional vitamin B_{12} deficiency is accompanied by combined homocystinuria and methylmalonic aciduria [3]. Homocystinuria type I is currently screened by a high blood concentration of methionine, another biochemical characteristic of this type, which is overproduced from homocysteine that accumulates in this disorder. Elevated plasma levels or increased urinary excretion of methionine are also found in isolated hypermethioninemia due to hepatic methionine adenosyltransferase (EC 2.5.1.6) (MAT) deficiency [1]. Most cases having MAT deficiency are, however, clinically free of symptoms; this result indicates that the accumulation of methionine in the body is not harmful.

Matsumoto and Kuhara have previously described a new method for the diagnosis of metabolic disorders using urease treatment of urine [4]. We describe, herein, a rapid and simple procedure for differential diagnosis of homocystinuria by analyzing the urine of patients.

2. Materials and methods

2.1. Patients

Case 1 was a 20-year-old male. He had skeletal abnormalities, dislocated optic lenses, mental retardation and sinus thrombosis characteristic of homocystinuria type I. He manifested megaloblastic anemia. His serum folate and vitamin B_{12} were found to be below the normal ranges. Therefore, he was treated with folate and vitamin B_{12} and the megaloblastic anemia disappeared. Urine samples obtained before and after treatment with folate and vitamin B_{12} were examined.

Case 2 was an 18-year-old female and the sibling of case 1. She had been enzymatically diagnosed as having homocystinuria type I.

Case 3 was a female aged 4 years and 1 month. Due to microcephaly and poor weight gain observed shortly after birth, she had been suspected as having homocystinuria which was verified enzymatically as 5,10-methylenetetrahydrofolate reductase deficiency. Although she was treated with folate, vitamin B_{12}, methionine, pyridoxine and betaine, she presented vomiting, convulsions, hypotonia and irritability. Urinary amino acid analysis using a conventional amino acid analyzer showed markedly increased cystathionine and homocystine and methionine within the normal range.

Case 4 was a 6-year-old male with homocystinuria type I.

2.2. Chemicals

Urease type C-3 was obtained from Sigma (St. Louis, MO, USA). Seven stable isotope-incorporated compounds were used as internal standards. [$^2H_4$]Cystine, [$^2H_8$]homocystine and [$^{15}N_2$]orotate were purchased from Cambridge Isotope Laboratory (Andover, MA, USA). [$^2H_3$]Methionine, [$^2H_3$]creatinine and [$^{15}N_2$]uracil were purchased from Isotec (Miamisburg, OH, USA). [$^2H_3$]Methylmalonate was purchased from MSD Isotopes (Pointe-Claire-Dorval, Quebec, Canada). The purity of stable isotope-incorporated compounds was >98% as judged by the lack of additional peaks on GC–MS. The purity of the stable isotope-incorporated compounds was also more than 99% except for uracil (98%).

2.3. Sample preparation

The procedure for sample preparation and GC–MS measurement was basically the same as previously described, which can be used for diagnosing a variety of metabolic disorders [4]. It includes urease treatment, alcohol deproteinization, evaporation to dryness, and trimethylsilylation. The urine (0.1 ml) was incubated with urease at 37°C for 10 min to decompose and remove excess urea present in the urine. In order to make accurate quantification, we used stable isotope-labeled internal standards. The amount of internal standard spiked into 100 μl of urine was 100, 10(5), 10, 10, 4(1), 4(1) and 10 nmol for creatinine, methionine, cystine, homocystine, uracil, orotate and methylmalonate, respectively. For the pilot study of neonatal screening, the amounts of some were reduced as shown in parentheses. Following deproteinization with ethanol, centrifugation to remove any precipitate, and evaporation to dryness, the residue was trimethylsilylated by adding 100 μl of a mixture of BSTFA and TMCS (10:1, v/v) and heating at 80°C for 30 min as described previously [4].

2.4. GC–MS analysis

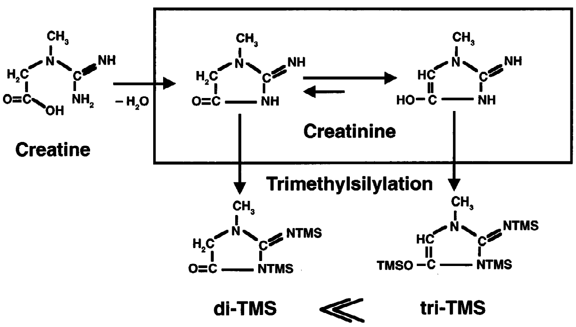
Aliquots (1 μl) of derivatized extract were injected into a GC–MS apparatus using an automatic injection mode with a split ratio of 1:40 (1:10–1:50). A bench-top HP GC-MSD (HP6890/MSD5973) was used for GC–MS measurement. Separation was carried out on a fused-silica DB-5 (30 m×0.25 mm I.D.) with a 0.25 μm film thickness of 5% phenylmethylsilicone (J&W, Folsom, CA). The oven temperature was programmed to increase at the rate of 17°C/min from 60 to 320°C with final holding for 10 min. After the set of analyses, the column oven temperature was kept at 300°C for 1–2 h to clean the column. The temperatures of the injection port and the transfer-line were 250 and 300°C, respectively, and a single tapered deactivated liner was used. Helium was used as carrier, with a flow-rate of 1.2 ml/min. Electron impact mass spectra were obtained by repetitive scanning at the scan rate of 2.5 cycles per s from m/z 50 to m/z 650. All other conditions for GC–MS measurements were the same as described previously [4].

2.5. Creatinine determination

During this analytical procedure, creatine as well as d_3-creatinine is almost completely converted to creatinine, and creatinine is quantitatively recovered, as previously described by Shoemaker and Elliott, who therefore used d_3-creatine as an internal standard [5] (see also Matsumoto and Kuhara [4]). In our experiments, using d_3-creatinine as an internal standard, the value of endogenous creatinine plus creatine is also obtained. The evaluation of metabolite levels relative to total creatinine in urine has been reported to be useful during clinical episodes of patients with metabolic disorders [6]. Trimethylsilylation of creatinine gave its tri-TMS derivative (major) and di-TMS (minor). 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 to directly quantify all the metabolites (Scheme 1). We also determined both creatinine and creatine by an auto-analyzer (COBAS FARA), and expressed the urinary metabolite levels relative to the creatinine value.

2.6. The mass spectra, isotope dilution method, and standard curves and intra-assay variability

Mass spectra of seven compounds (unlabelled; upper) and their stable isotope-incorporated internal standards (lower) are shown in Fig. 2 (a–g). Quantitation was performed by the relative peak area of the target ions between each compound and its internal standard. The ions used for targets were 247/250 for methylmalonate, 241/243 for uracil, 176/179 for methionine, 329/332 for creatinine, 254/256 for orotate, 411/415 for cystine and 278/282 for homocystine. To quantify metabolites in urine samples, calibration lines were obtained from mass chromatograms. Various amounts of authentic compounds were added to 100 μl of saline spiked with fixed amounts of their internal standards, and these mixtures were processed as described above and analyzed by GC–MS. Amount of internal standard spiked into 100 μl of saline was 100, 10(5), 10, 10, 4(1), 4(1) and 10 nmol for creatinine, methionine, cystine, homocystine, uracil, orotate and methylmalonate, respectively. For the pilot study of neonatal screening, the amounts of some internal standards were reduced from one-fourth to one-tenth as shown in parentheses. The correlation coefficients of the calibration lines were 0.9998 ($y=10.13x$) for methylmalonate, 0.9991 ($y=3.52x$) for uracil and 0.9991 ($y=3.71x$) for orotate. An additional correction was made for the quantitation of uracil and orotate, because endogenous (due to natural abundance) and labelled compounds (due to the presence of unlabelled ones) both significantly contribute the intensities of the other ions to each other. The isotope dilution method for the determination of the three amino acids, as previously reported by Schulman and Abramson [7], and for that of creatinine gave good quantitative data, as shown in Fig. 3. The intra-assay variability was obtained by repeated GC–MS analysis of a derivatized sample from urine of a patient with type I (case 4) ($n=8$). The values of C.V. (%) were small; 1% for creatinine, 2% for uracil and methionine, 3% for orotate and homocystine, except for cystine (10%) and methylmalonate (5%), probably due to the relatively low intensity of the target ion [M-COOTMS] at m/z 411 and [M-CH_3] at m/z 247, respectively.



Scheme 1. Determination of urinary creatinine using stable isotope-labelled internal standard.

3. Results and discussion

Inborn errors of cysteine metabolism, most of which cause severe diseases, can be detected by the presence of an abnormal accumulation in body fluids of metabolites specific to each disorder. The pathological consequences in homocystinuria types I–III can be prevented or significantly reduced by appropriate intervention in the neonatal or infantile periods. Consequently, practical, sufficiently specific and cost-effective neonatal screening programs are currently conducted in developed countries. As shown in Table 2, all neonates born in Japan are screened for homocystinuria type I by targeting methionine in the dried blood spots on filter paper,

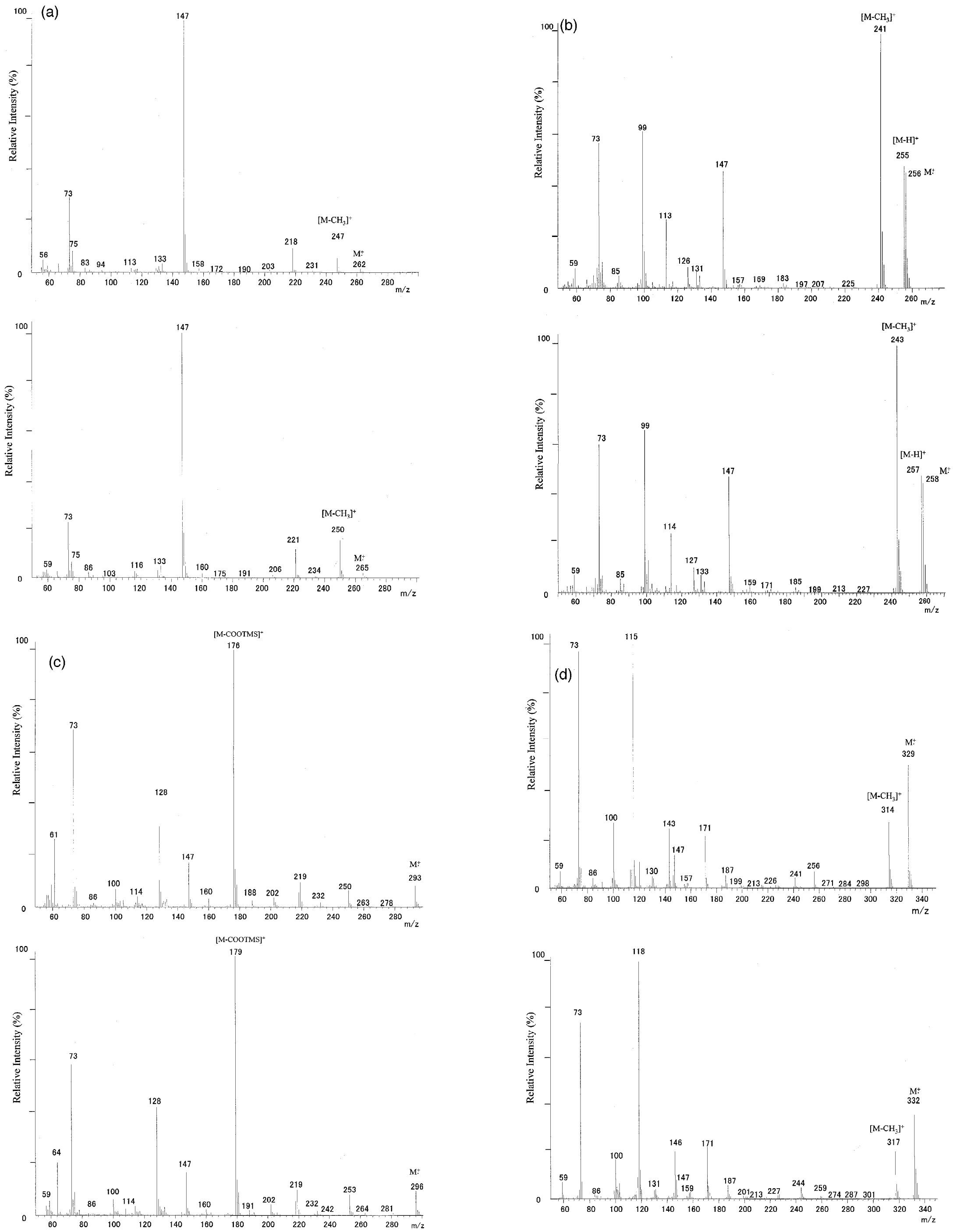


Fig. 2. Mass spectra of trimethylsilyl derivatives of authentic compounds (upper) and their stable isotope-labeled internal standards (lower). (a) methylmalonate and methylmalonate(methyl-d_3), di-TMS, (b) uracil and $^{15}N_2$-uracil, di-TMS, (c) methionine and methionine (methyl-d_3), di-TMS, (d) creatinine and creatinine(methyl-d_3), tri-TMS. (e) orotate and $^{15}N_2$-orotate, tri-TMS, (f) cystine and 3,3,3′,3′-d_4-cystine, tetra-TMS, (g) homocystine and 3,3,3′,3′,4,4,4′,4′-D_8-homocystine, tetra-TMS.

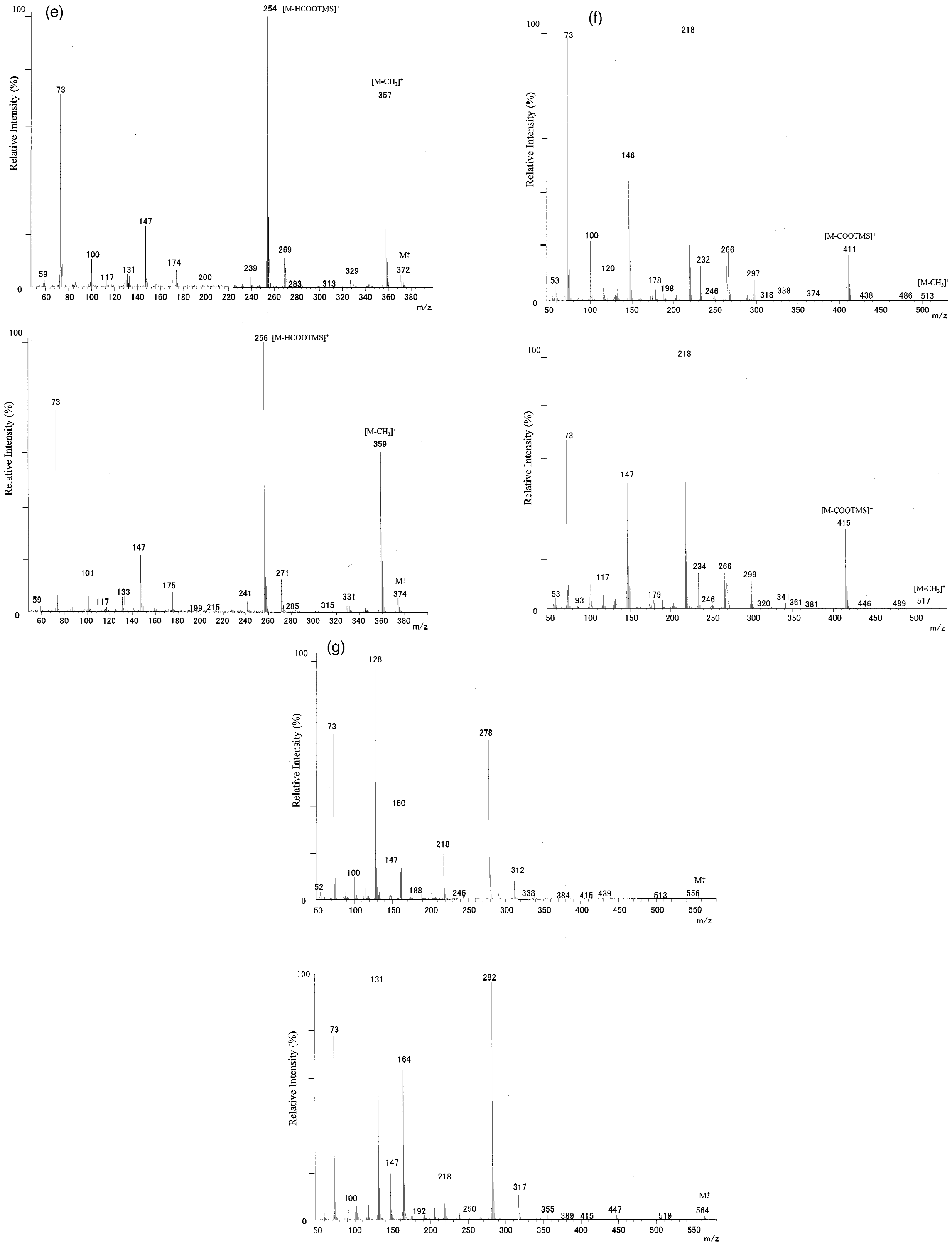


Fig. 2. (*continued*)

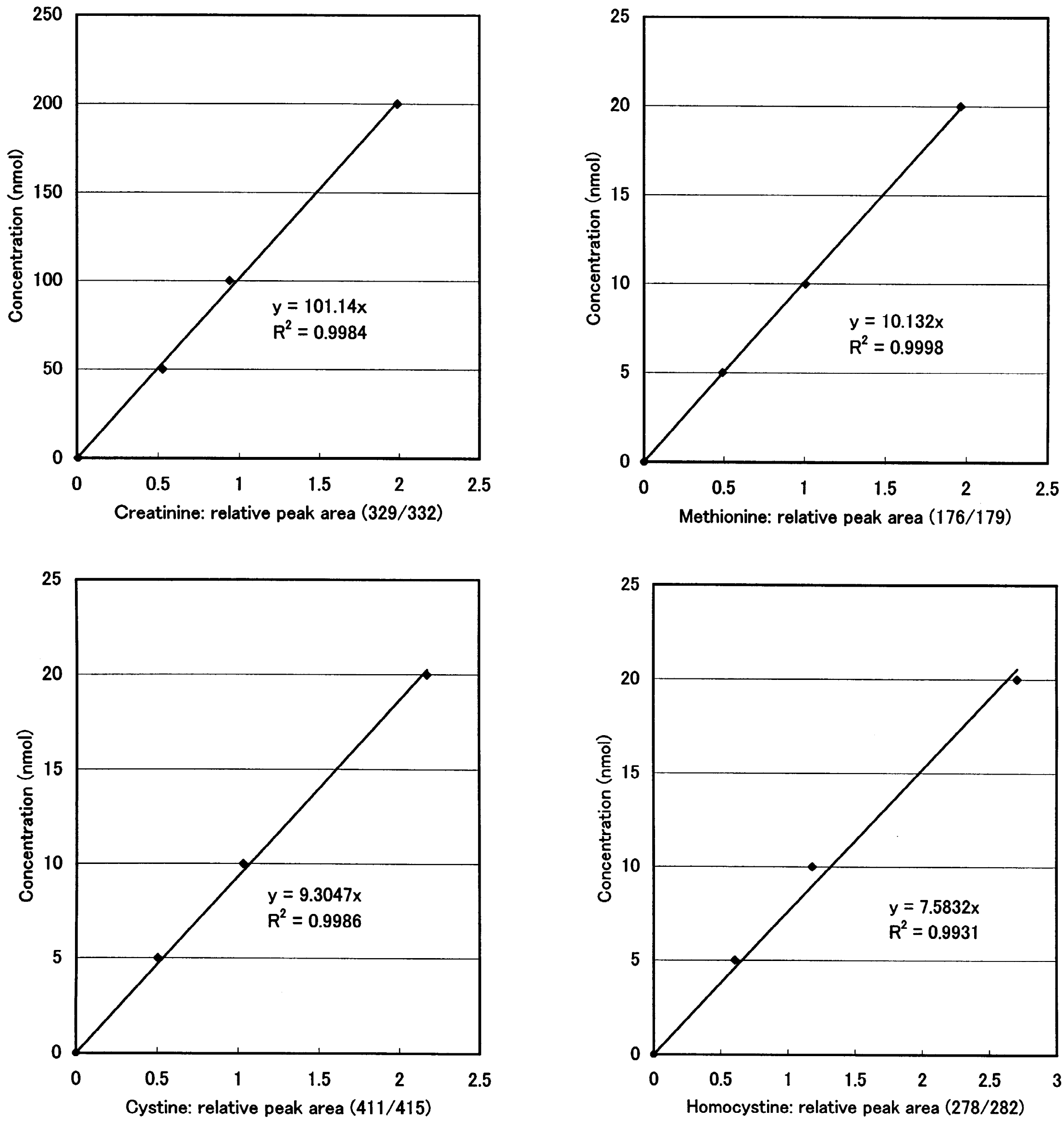


Fig. 3. Calibration lines for measurement of metabolites using stable isotope dilution-GC–MS. Amount of internal standard spiked in 100 μl of saline was 100, 10, 10, 10 nmol for creatinine, methionine, cystine and homocystine, respectively.

using semiquantitative bacterial inhibition assay ('the Guthrie tests'). Recently, a tandem mass spectrometric (MS–MS) method has been developed to analyze methionine, other amino acids and acylcarnitines from dried blood spots in a single test [8]. In this isotope dilution-MS–MS method, homocystinuria is screened as hypermethioninemia, and the latter is evaluated as the ratio of methionine to leucine plus isoleucine in the blood. Therefore, in both methods, homocystinuria type I and isolated hypermethioninemia are detected, and homocystinuria type II is not screened (Table 1).

Urinary water-soluble organic compounds are the end-products or intermediates of the catabolism of amino acids, sugars, lipids and many other endogenous compounds. Moderately increased methylmalo-

Table 1
Target compounds in four methods for the screening for homocystinuria

Type of analysis	Point	Line	Line	Planar
Compounds measured	One compound	A series of organic acids	Series of acylcarnitines and amino acids	Multi-categories of compounds
Sample	Blood	Urine[b]	Blood	Urine[c]
Method or instrument	BIA[a]	GC–MS	MS–MS	GC–MS
Target and evaluation	Met	Methylmalonate	Met/Leu+Ile	Methionine Homocystine Methylmalonate Orotate Uracil Cystine
Diseases screened or diseases diagnosable	Homocystinuria type I Hypermethioninemia	Methylmalonic aciduria due to homocystinuria type III	Homocystinuria type I Hypermethioninemia	Homocystinuria types I, II, III

[a] BIA, bacterial inhibition assay.
[b] Expressed as relative to creatinine.
[c] Expressed as relative to creatinine plus creatine.

nate excretion detected by conventional GC–MS analysis of urinary organic acids suggests the presence of homocystinuria type III abnormalities at the later steps in the biosynthesis of deoxyadenosylcobalamin, a coenzyme of methylmalonyl-CoA mutase, or vitamin B_{12}-responsive methylmalonic aciduria due to a methylmalonyl-CoA mutase apoenzyme abnormality (see Fig. 1). The former is confirmed by the concomitant urinary increase of homocystine detectable by a separate amino acid analysis, using a conventional automated amino acid analyzer. Since the discovery of isovaleric aciduria by Tanaka et al. in 1996 [9], GC–MS techniques have become indispensable for high-risk screening of organic acidurias [10,11]. For low-risk screening of large populations, however, only a limited number of projects presently implement GC–MS. A mass screening program for neuroblastoma at 3 weeks of age in Quebec adopted GC–MS analysis of urinary acids [12], and has been further extended to the screening for 20 or more different metabolic conditions [13]. Very recently, a GC–MS–MS screening method for 10 organic acidurias from urine specimens was described [14], in which 14 markers were quantified after solid-phase extraction, oximation/trimethylsilylation (90 min for derivatization) and a very short GC–MS–MS measurement (10 min). As they used the solid-phase extraction method, analyses were focused on organic acids. The polar organic acids, such as methylcitrate, a reliable index for propionic acidemia, were not targeted [14].

Shoemaker and Elliott reported in 1991 that, after excessive urea in the urine is degraded with urease and removed, urinary organic acids, amino acids and sugars can be analyzed simultaneously using GC–MS, and d_3-creatine can be used as an internal standard [5]. Their idea to use urease as well as the recent advances in GC–MS instrumentation and software opened a new analytical approach for the study of inherited and acquired metabolic disorders. Shoemaker's procedure for sample pretreatment, however, takes several hours, needs skilled technicians, and is not very practical for screening. Based on our experiences on chemical diagnosis of heritable disorders using GC–MS for more than two decades, we drastically modified and simplified Shoemaker's procedure for multiple sample analysis or for potential use in neonatal mass screening [4]. Our procedure takes 1 h for pretreatment of one sample or 3 h for a batch of 30 samples, plus 15 min for GC–MS measurement per sample.

The simple and accurate differential diagnosis of homocystinuria types I–III is critical, because the treatment is different for each type of disease. We had previously carried out the chemical diagnosis of homocystinuria using GC–MS, for organic acids analysis, and an automated amino acid analyzer. In the present study, we used the same urine specimens from three cases from two families of type I, and one case of type II, to examine whether this modified procedure, with the use of additional stable isotope-incorporated internal standards, enabled us to clearly distinguish or differentiate these disorders. As shown in Table 2, it becomes easy and rapid to make the differential diagnosis by simultaneously analyzing three amino acids, methylmalonate and two pyrimidine derivatives, with the use of the respective stable isotope-incorporated internal standards. In all patients with type I, homocystine and methionine were significantly increased, whereas methylmalonate was within the normal range, and cystine was decreased due to a cystathionine β-synthase deficiency.

In Figs. 4 and 5, the TIC and mass chromatograms of the trimethylsilyl derivatives of metabolites from a patient with type I (case 1) are shown. Case 1, now 20 years old, had developed skeletal abnormalities, dislocated optic lenses, mental retardation and sinus thrombosis characteristic of homocystinuria type I. As he temporarily manifested megaloblastic anemia, and his serum folate and vitamin B_{12} were below the normal ranges, he was treated with folate and vitamin B_{12}. Clinical and biochemical abnormalities were both ameliorated: megaloblastic anemia disappeared, homocystine decreased and methionine increased, as shown in Table 3. Orotate, that was markedly increased along with folate deficiency, decreased into the normal range after treatment with folate and vitamin B_{12}. Conversion of dUMP to dTMP, catalyzed by thymidylate synthase, is folate-dependent, and pyrimidine biosynthesis is regulated by end-product inhibition. Folate deficiency thus causes impaired DNA synthesis, enhanced pyrimidine biosynthesis, orotic aciduria and megaloblastic anemia. Orotate is the only intermediate in pyrimidine biosynthesis which is analyzable with the present procedure, using urine as the specimen. Because this patient never developed convulsions, he had never received anticonvulsants, which have a tendency to induce secondary folate deficiency. Because he appeared not to have malnutrition, the reason why he developed the deficiency of folate and/or vitamin B_{12} is unclear, but is very interesting. Increased remethylation of cysteine to form methionine in this condition may have the tendency to induce secondary deficiency of folate or vitamin B_{12}. Deficiency of folate appeared to induce more severe biochemical and clinical abnormalities than deficiency of B_{12}, and orotate appeared to be a valuable index for evaluation of folate deficiency induced secondarily in homocystinuria. For the screening of urea cycle disorders, except for carbamoylphosphate synthase deficiency and *N*-acetylglutamate synthase deficiency, our method is valuable as it targets orotate and uracil. Isolated orotic aciduria can also be differentiated from the other conditions, based on the concomitant increase of homocystine and methionine (type I) and that of methionine and cystine (type II), as shown in Table 2. In case I, folate supplementation significantly reduced the level of homocystine and dramatically increased that of methionine.

Another form of folate, 5-methyltetrahydrofolate (CH_3–H_4 folate), is the cofactor of methionine synthase for conversion of homocystine to methionine. Reduction of 5,10-methylenetetrahydrofolate (CH_2–H_4 folate) to CH_3–H_4 folate by methylenetetrahydrofolate reductase (MTHFR) is the only route for synthesis of the CH_3–H_4 folate that is utilized for conversion of homocystine to methionine. Deficiency of MTHFR is the cause of homocystinuria type II, the most common inborn error of folate metabolism. In case 3

Table 2
Different conditions in trans-sulfuration and DNA synthesis

Type	Methionine	Homocystine	Methylmalonate	Orotate	Uracil	Cystine
I	↑	↑↑	–	–	–	↓
II	↓ or –	↑	–	–	–	↑
III	↓ or –	↑	↑	–	–	–
Folate deficiency	↓ or –	↑	–	↑	–	–
B_{12} deficiency	–	↑	↑	–	–	–

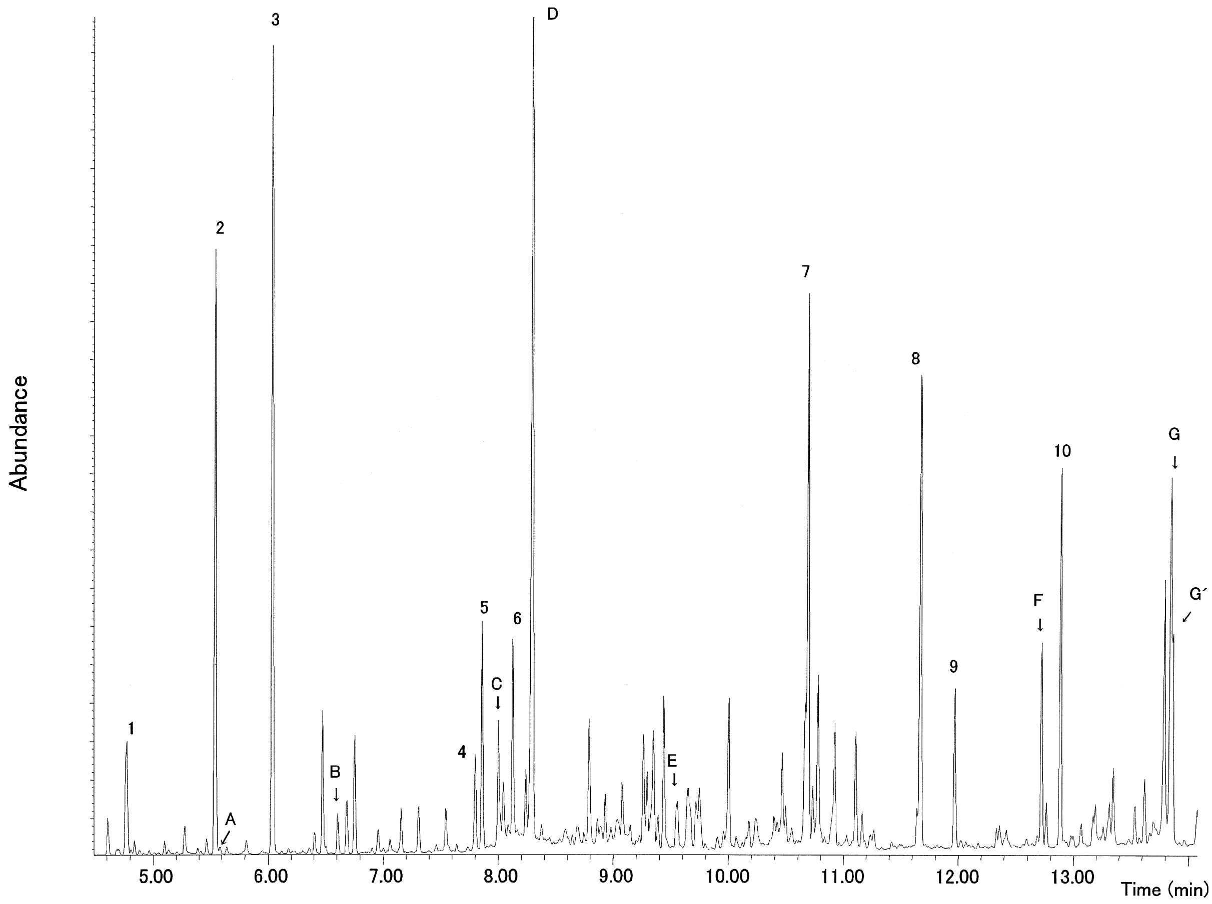


Fig. 4. TIC chromatograms of trimethylsilyl derivatives of metabolites from the urine of a patient with homocystinuria type I (case 1) during transient megaloblastic anemia due to folate and vitamin B_{12} deficiency. Peak identifications are: (1) glycine; (A) methylmalonate and I.S.; (2) aminobutyrate; (3) phosphate and leucine; (B) uracil and I.S.; (4) erythritol; (5) threitol; (C) methionine and I.S.; (6) tetronate; (D) creatinine and I.S.; (E) orotate and I.S.; (7) mannitol; (8) urate; (9) *n*-heptadecanoate spiked; (F) cystine and I.S.; and (10) pseudouridine; (G) homocystine-d_8 (I.S.); (G′) homocystine.

(severe type II), homocystine was still significantly increased even under intensive treatment with folate, vitamin B_{12}, methionine, pyridoxine and betaine. Recently it was demonstrated that homozygosity of a polymorphism, A222V, is the most common genetic cause of mild homocysteinemia and is a risk factor for the development of cardiovascular disease. From the structural analysis of MTHFR in this condition, a new role of folate in the specific activity and thermolability of MTHFR was demonstrated [15], and, in these homozygotes, a preferential supply of folate for DNA synthesis and an accumulation of homocysteine under low folate conditions was suggested [15].

Homocystinurias are characterized by different etiologies and different therapeutic measures. Therefore, rapid differential diagnosis is critical in order to ensure effective treatment. The simple procedure presented here is useful for diagnosis of homocystinuria, isolated hypermethioninemia, methylmalonic acidemia and isolated orotic aciduria, for monitoring the biochemical and nutritional con-

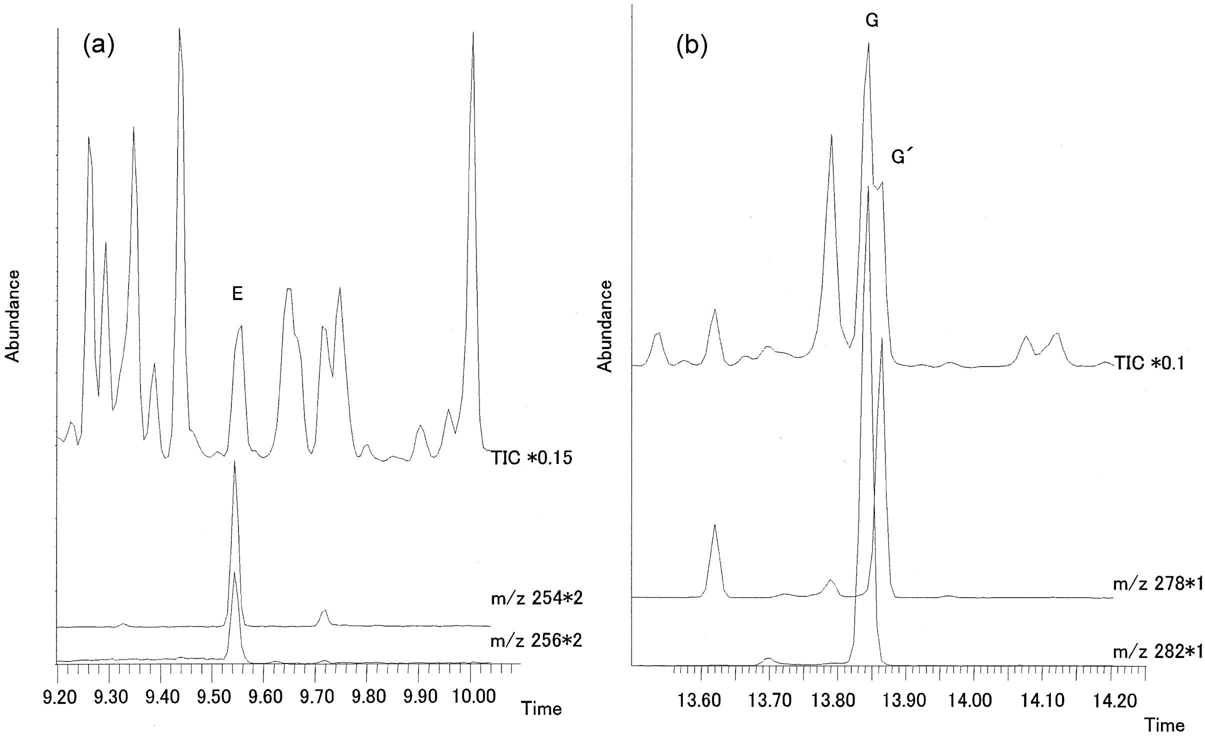


Fig. 5. Partly shown TIC and mass chromatograms of Fig. 4: (a) for orotate, (b) for homocystine.

Table 3
Urinary metabolite levels in patients with homocystinuria[a]

Case	Hcys	Met	Cys	MMA	Uracil	Orotate	Creat	Cystathionine[b]	Folate[c]	Vit. B_{12}[c]
1	26.36	11.82	0.97	1.03	8.85	9.60	8.27	0.00	1.20	0.16
Before[d]	(25.53)	(11.45)	(0.94)	(1.00)	(8.58)	(9.30)	(8.54)			
1	4.35	30.73	2.07	0.48	4.66	0.43	21.66	0.00	41.90	0.53
After[d]	(4.17)	(29.47)	(1.99)	(0.46)	(4.46)	(0.41)	(22.59)			
2	21.09	12.55	1.35	2.04	3.38	2.16	18.69	0.20	4.40	0.17
	(20.18)	(12.01)	(1.30)	(1.95)	(3.67)	(2.06)	(19.53)			
3	4.46	16.52	14.78	1.55	13.00	0.91	7.13	15.60	ND	ND
	(1.99)	(7.37)	(6.60)	(0.69)	(5.80)	(0.41)	(15.99)			
Control mean	UD	3.00	7.70	1.83	11.80	0.83	6.36	0–3.4	2.4–9.8 (range)	0.25–0.94
4Y–23Y	(UD)	(1.63)	(4.36)	0.92	(6.61)	(0.46)	(10.57)			
SD	UD	1.58	4.84	0.94	7.77	0.69	3.13			
n=27	(UD)	(0.76)	(2.94)	(0.45)	(3.89)	(0.36)	(3.57)			

[a] Hcys, homocystine; Met, methionine; Cys, cystine; MMA, methylmalonate; Creat, creatinine; UD, undetectable; and ND, not determined. Values are expressed as mmol per mol creatinine except for folate and vitamin B_{12}. The creatinine concentration was obtained by autoanalyzer and by GC–MS in parentheses (see Section 2). Case 1 (type I), a 20-year-old male; case 2, the sibling of case 1 (18-year-old female); case 3 (type II), a 4-year-old girl. For creatinine, values are expressed as µmol/ml.

[b] Cystathionine was determined by using a conventional amino acid analyzer.

[c] Folate and vitamin B_{12} were measured by routine laboratory test (ng/ml in serum).

[d] Case 1 developed megaloblastic anemia and the values were obtained before and after treatment with folate and vitamin B_{12}.

ditions of the patients (especially for acquired folate and vitamin B_{12} deficiency) and for evaluating the efficacy of treatment.

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