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Simple gas chromatographic–mass spectrometric procedure for diagnosing pyrimidine degradation defects for prevention of severe anticancer side effects

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Abstract

Inborn errors of pyrimidine degradation, dihydropyrimidine dehydrogenase deficiency and dihydropyrimidinase deficiency, are less rare than has generally been assumed. Many asymptomatic cases have been reported, and in patients with symptoms, the clinical abnormalities are variable and nonspecific. Withdrawal of pyrimidine analogues such as 5-fluorouracil (5FU), a commonly used anticancer drug, from the cancer chemotherapy regimens of patients with pyrimidine degradation deficiencies, however, is critical because 5FU is degraded in vivo by pyrimidine-degradative 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 upon administration of such medication. Therefore, a rapid and specific method for identifying cancer patients with these enzyme deficiencies prior to treatment with 5FU is critical. To address this problem, we established methods for highly sensitive yet specific determinations of thymine, uracil, dihydrothymine, dihydrouracil, orotate and creatinine simultaneously in 0.1-ml liquid urine or filter-paper urine. This method involves stable isotope dilution, a simplified urease treatment previously described and gas chromatography–mass spectrometry without prior fractionation. The high recovery and low C.V. values were obtained and healthy control values were also determined for these metabolites. Using artificially prepared urine specimens simulating these disorders, the chemical diagnosis can be made clearly, and no further analysis appears to be required for differential chemical diagnosis.

Keywords: Dihydropyrimidine dehydrogenase deficiency; Dihydropyrimidinase deficiency; 5-fluorouracil; Pyrimidine; Cancer chemotherapy

1. Introduction

Inborn errors of pyrimidine degradation are less rare than has generally been assumed, and clinical presentations of such patients are variable and nonspecific [1–3]. Pyrimidines are degraded in humans in four steps catalyzed by dihydropyrimidine dehydrogenase (DHPDH, EC 1.3.1.2), dihydropyrimidinase (DHP, EC 3.5.2.2), β-ureidopropionase (UP, EC 3.5.1.6), and three aminotransferases, as shown in Fig. 1. Patients with DHPDH deficiency (MIM 274270) do not exhibit a characteristic clinical phenotype [2,4] and those with DHP deficiency (MIM 222748) also show a variable clinical phenotype consisting of seizures or epileptic attacks, or mental retardation [5,6]. Asymptomatic infants and

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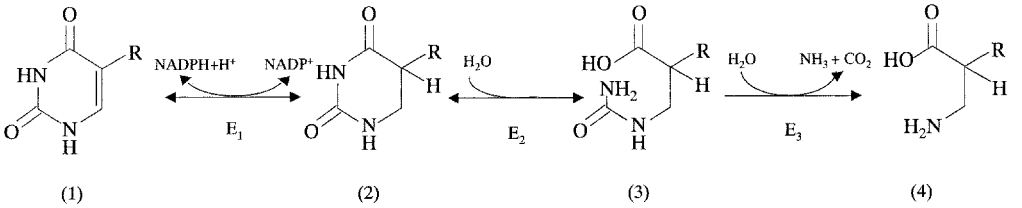


Fig. 1. The degradative pathway of pyrimidines. E1: dihydropyrimidine dehydrogenase, E2: dihydropyrimidinase, E3: β-ureidopropionase, [*R*(−)3-amino-2-methylpropionate-pyruvate aminotransferase (D-3-aminoisobutyrate-pyruvate aminotransferase), β-alanine-pyruvate aminotransferase, 4-aminobutyrate aminotransferase] R = H: Uracil (1), 5, 6-Dihydrouracil (2), β-Ureidopropionic acid (3), β-Alanine (4); R = CH_3: Thymine (1), 5, 6-Dihydrothymine (2), β-Ureidoisobutyric acid (3), β-Aminoisobutyric acid (4); R = F: 5-Fluorouracil (1), 5, 6-Dihydro-5-fluorouracil (2), α-fluoro-β-ureidopropionic acid (3), α-fluoro-β-alanine (4).

adults with DHP deficiency have also been reported [3,7]. No treatment specific for these enzyme defects has been described, but withdrawal of pyrimidine analogues such as 5-fluorouracil (5FU), a commonly used anticancer drug, from cancer chemotherapy regimens of patients with these defects is critical. These patients suffer from severe neurotoxicity, sometimes leading to death, following administration of 5FU [8–10], because 5FU is degraded in vivo by DHPDH DHP, and β-ureidopropionase to fluorinated β-alanine (70–80% of administered dose) [10]. As previously asymptomatic cases may also develop onset of severe clinical symptoms upon administration of such medication, presymptomatic diagnosis by determining thymine, uracil, dihydrothymine and dihydrouracil in urine samples is critically important.

Several methods to screen for disorders of pyrimidine metabolism that use HPLC [11,12], two dimensional thin-layer chromatography [13], amino acid analysis of urine before and after acid hydrolysis [14], gas chromatography, or gas chromatography–mass spectrometry (GC–MS) [15] have been reported. To date, however, these methods have either had difficulty for quantitation, lacked sensitivity or specific system of detection or needed further analysis to establish a differential diagnosis of these disorders. A rapid and simple procedure extending the chemical diagnostic capacity to new categories of metabolic disorders was proposed by Matsumoto and Kuhara [16]. This procedure drastically simplified and shortened the method described by Shoemaker and Elliott [17]. Although sample preparation including derivatization is required prior to GC–MS analysis and analytical time for GC–MS is longer than that for HPLC, whole mass spectra and retention time information of various categories of compounds in urine are stored. Therefore mostly no further analysis is required for differential chemical diagnosis. We investigated to identify either symptomatic or asymptomatic homozygotes and heterozygotes in the pyrimidine degradative pathway by simultaneously determining thymine, uracil, dihydrothymine, dihydrouracil, orotate and creatinine in 0.1-ml liquid urine or filter-paper urine. Here we describe a method that involves stable isotope dilution, simplified urease treatment previously described and gas chromatography–mass spectrometry without prior fractionation.

2. Materials and methods

2.1. Chemicals

1, 3-$^{15}N_2$-uracil and creatinine-methyl-$^{2}H_3$ (d_3-creatinine) were purchased from Isotec (Miamisburg, OH, USA) and 1, 3-$^{15}N_2$-orotate from Cambridge Isotope Laboratory (Andover, MA, USA). The purity of the three stable isotope-labeled compounds, used as internal standards, was higher than 99%, except for uracil (98%), as judged by the lack of additional peaks on GC–MS. Thymine, 5,6-dihydrothymine (DHT), 5,6-dihydrouracil (DHU), 5-fluorouracil (5FU) and urease type C-3 were obtained from Sigma (St. Louis, MO, USA), orotate and creatinine from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan) and uracil from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

2.2. Sample preparation and GC–MS analysis

The procedures for sample preparation and GC–MS measurement were basically the same as previously described, and can be used for diagnosing a variety of metabolic disorders [16]. One-tenth of a milliliter of liquid urine or a urine-soaked filter paper strip was pretreated with urease at 37°C for 10 min to degrade and remove excess urea present in urine. For accurate quantification, 4, 4 and 100 nmol of stable isotope-labeled uracil, orotate and creatinine, respectively, were spiked into 0.1 ml of urine. The labeled uracil was also used as the internal standard for the quantification of uracil, thymine, DHT and DHU. After deproteinization of the sample 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 [16]. In this sample preparation procedure, urinary creatinine is quantitatively recovered and the content of endogenous creatinine plus creatine is obtained because creatine is almost completely converted to creatinine [16,17]. Measurement of creatinine and creatine separately was also done using an auto-analyzer (Beckman CX5), and the urinary metabolite levels were evaluated on the basis of both standards: creatinine and creatinine plus creatine.

2.3. GC–MS analysis

Aliquots (0.5 or 1 μl) of derivatized extracts were injected into a bench-top HP GC-MSD (HP6890/MSD5973) using an automatic injection mode with a split ratio of 1:30 (1:10-1:50). All the conditions for GC–MS measurement were the same as described previously [16].

2.4. Mass spectra, standard curves and intra-assay variability

Mass spectra of target compounds and internal standards are shown in Fig. 2(1)–(10). The ions used for targets in the quantitation were m/z 241 and 255 for uracil, m/z 255 and 270 for thymine, m/z 271 and 257 for DHT, m/z 243 and 257 for DHU, m/z 329 and 115 for creatinine and m/z 254 and 357 for orotate. To quantify metabolites in urine samples, calibration curves were obtained from mass chromatograms. Various amounts of authentic compounds were added to 100 μl-urine specimens spiked with fixed amounts of the internal standards, and these mixtures were processed as described above and analyzed by GC–MS. The correlation coefficients of the calibration curves were 0.998 for uracil and thymine and 0.999 for DHT and DHU. The correction was also made for the quantitation of uracil, orotate and creatinine because endogenous (due to natural abundance) and labeled compounds (due to the presence of unlabeled ones) both contribute significantly to the intensities of the other ions. The reproducibility of the quantitation was determined by analysis of multiple sample preparations ($n=5$) and GC–MS analysis of derivatized samples from a urine specimen. The values of C.V. (%) were 3% for uracil and orotate, 4% for thymine and creatinine, and 5% for DHT and DHU. The intra-assay variability of GC–MS measurement was obtained by single sample preparation ($n=1$) and repeated GC–MS analysis ($n=9$) of derivatized samples from urine. The values of C.V. (%) were small: 1% for creatinine, 2% for uracil and orotate, 4% for thymine and 5% for DHU, but slightly greater for DHT (7%) at low concentrations, but these were improved: 3% for thymine, 4% for DHU and 5% for DHT, at high concentrations.

In order to simulate results for typical and moderate cases and for heterozygotes, of DHPDH deficiency and DHP deficiency, artificial specimens were prepared by spiking various amounts of uracil, thymine, DHU and DHT into a urine specimen, as shown in Table 1.

3. Results and discussion

The pathological consequences of inborn errors of pyrimidine degradation are variable and nonspecific, and the disorders appear to be less rare than has generally been assumed. Since the original description of DHPDH deficiency [4], at least 33 cases have been reported, and the existence of asymptomatic cases has also become clear [1,2]. Among the seven cases with DHP deficiency described, two had mental retardation, but two each of infant and adult

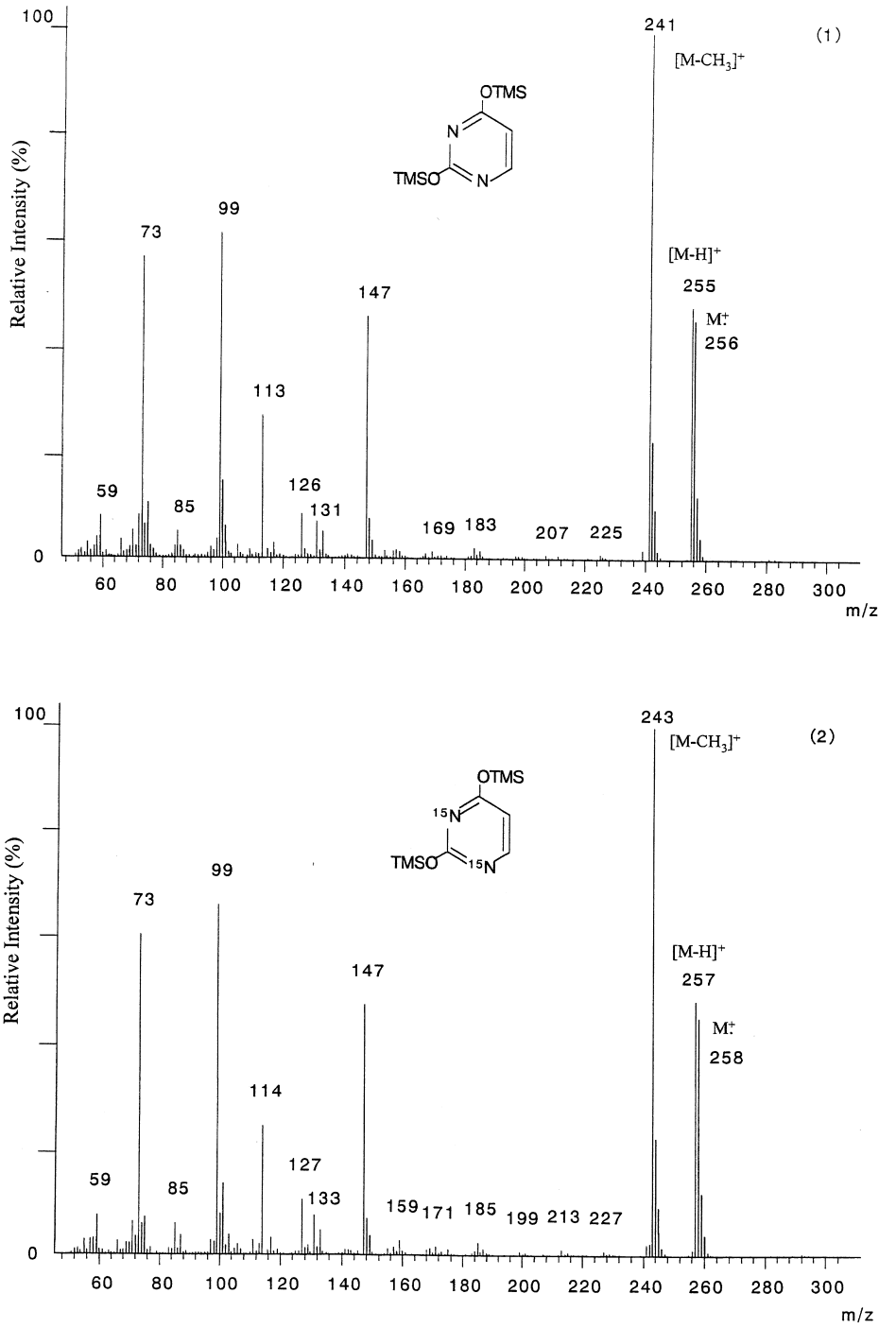
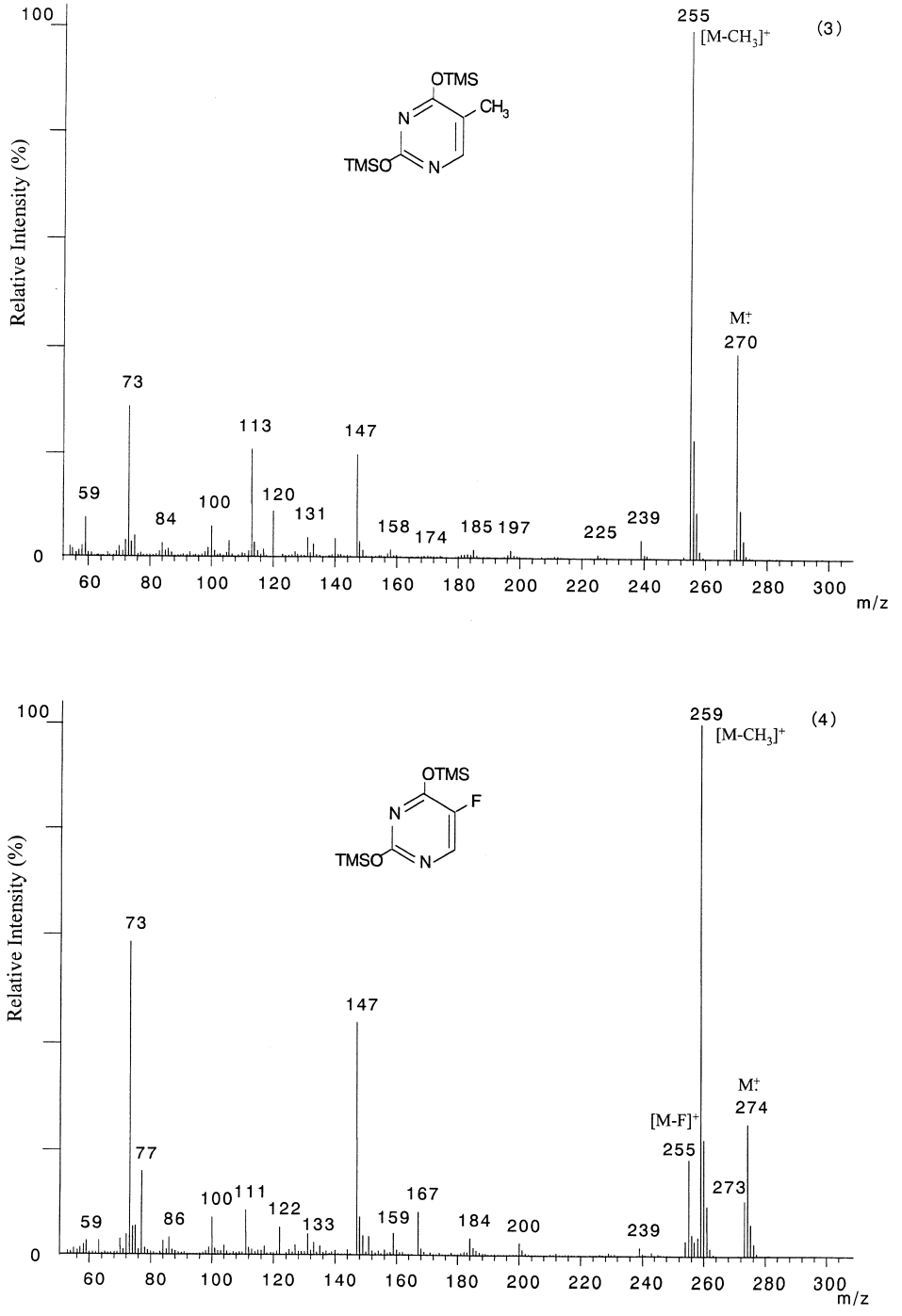


Fig. 2. Mass spectra of trimethylsilyl derivatives of authentic compounds and their stable isotope-labeled internal standards (IS). 1. uracil di-TMS, 2. $^{15}N_2$-uracil di-TMS (IS), 3. thymine di-TMS, 4. 5-fluorouracil di-TMS, 5. 5, 6-dihydrothymine di-TMS, 6. 5, 6-dihydrouracil di-TMS, 7. creatinine tri-TMS, 8. creatinine (methyl-d_3) tri-TMS, 9. orotate tri-TMS, 10. $^{15}N_2$-orotate tri-TMS.



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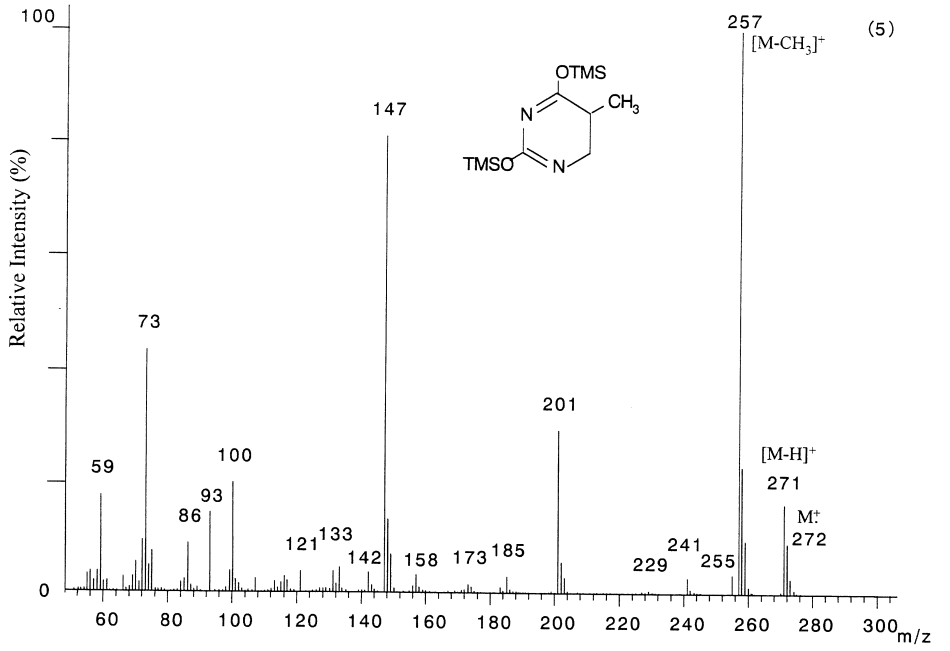

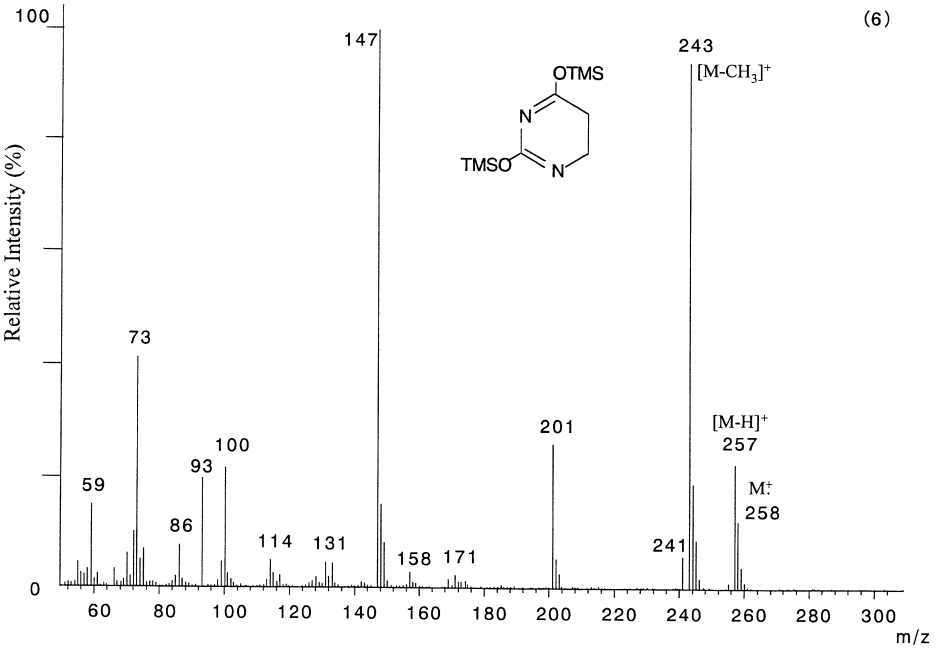

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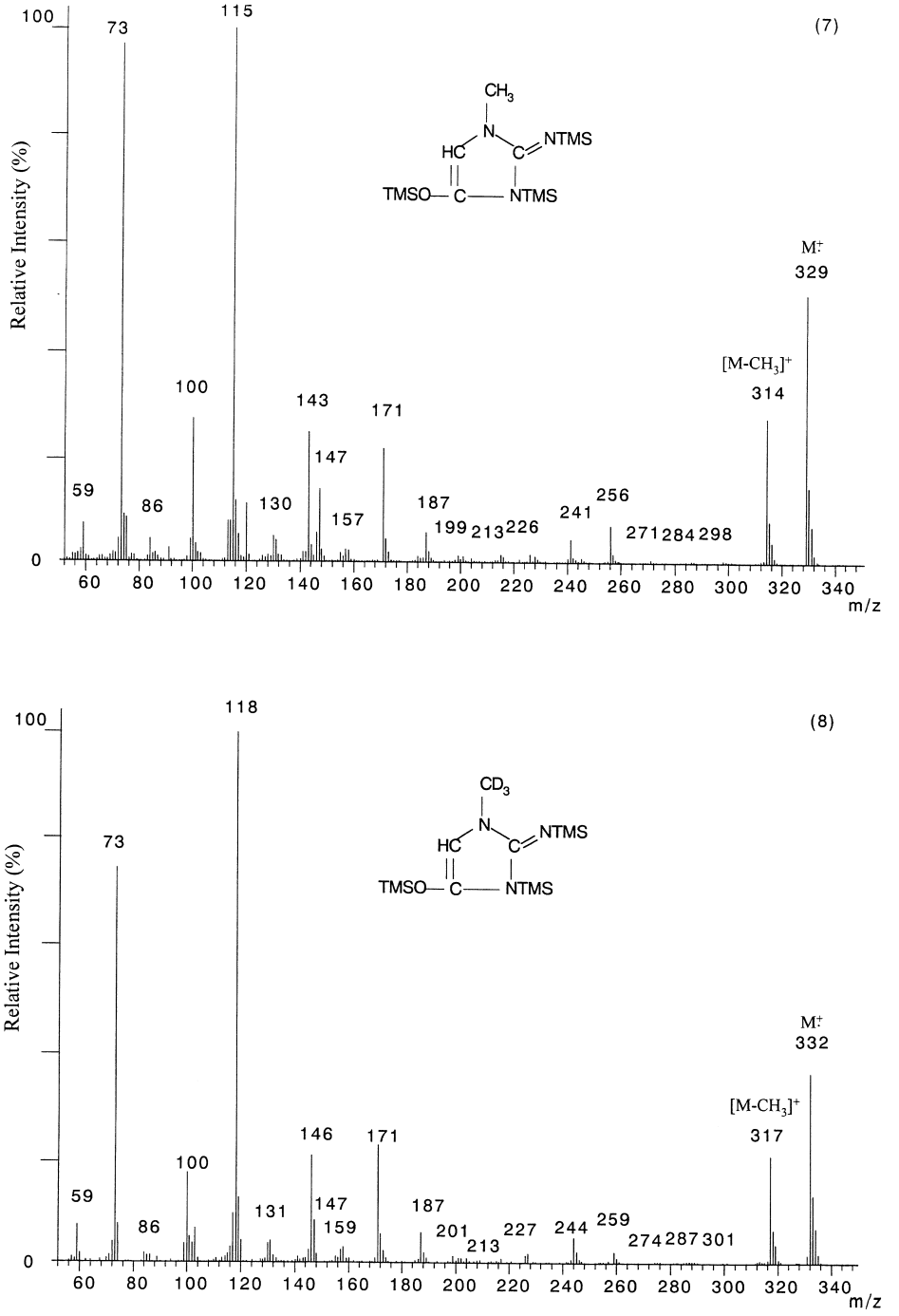

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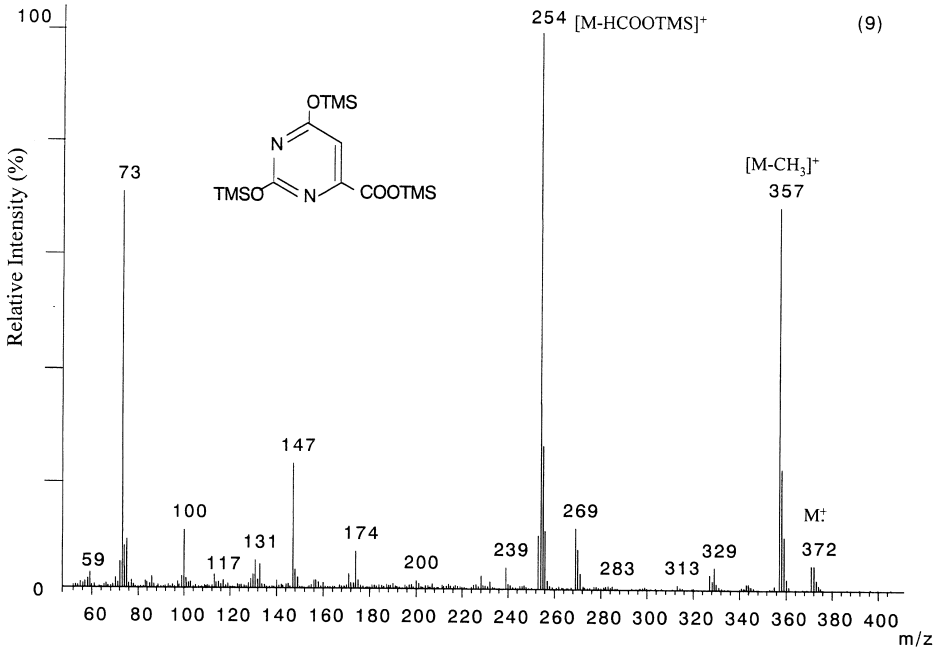

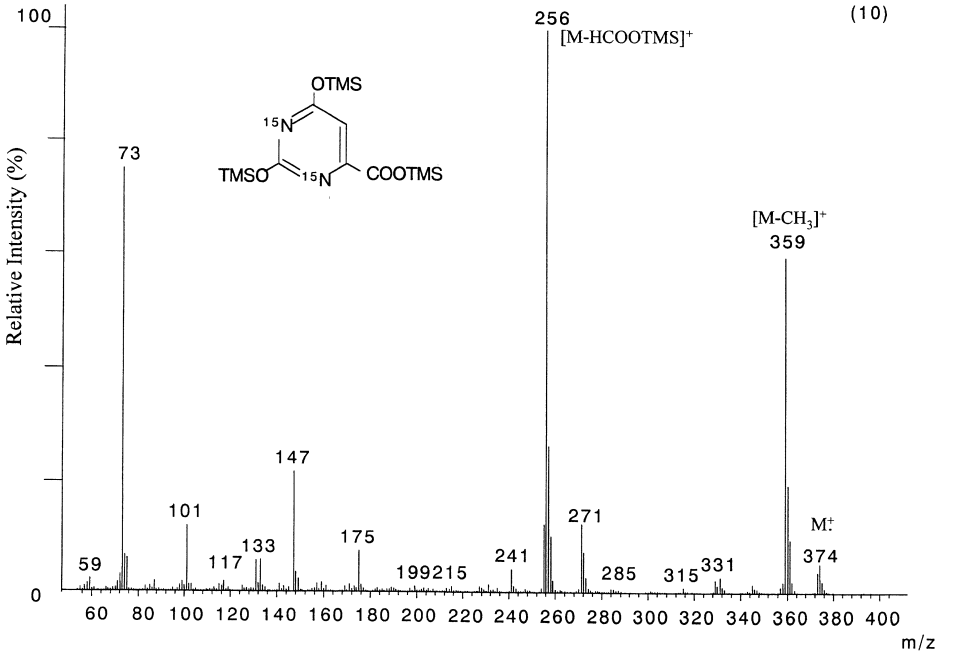

Fig. 2. (*continued*)

Table 1
Artificial urine specimens simulating DHPDH deficiency and DHP deficiency[a]

Amounts spiked	Uracil	Thymine	DHU	DHT
None	–	–	–	–
Small amounts	8	4	8	4
DHPDH heterozygote	16	8	–	–
DHPDH moderate	80	40	–	–
DHPDH typical	160	80	–	–
DHP heterozygote	8	4	16	8
DHP moderate	16	8	80	40
DHP typical	32	16	160	80

[a] Simulation was based on Ref. [3]. Values are expressed as nmol spiked into 100 μl of urine. A urine specimen containing 0.341 μmol creatinine and 0.023 μmol creatine per 100 μl was taken from a male aged 5 years and 4 months who had no metabolic disorders. This sample was spiked in the present experiments. For healthy controls above 4 years of age, the mean values of urinary creatinine and creatine were 0.83 and 0.29 μmol per 100 μl, respectively.

asymptomatic cases were also discovered by using HPLC [3,7,9]. No treatment specific for the enzyme defects has been described, but withdrawal of 5FU from cancer chemotherapy regimens of individuals with such deficiencies is critical [8–10]. 5FU toxicity occurs in heterozygotes as well as homozygotes with DHPDH deficiency [10,20]. It has also been speculated that DHP homozygotes may have a high risk of 5FU toxicity [3]. Thus, the detection of homozygotes and heterozygotes among cancer patients prior to the introduction of 5FU is critical in order to prevent severe side effects of this drug. Consequently, practical, sufficiently specific and rapid chemical diagnosis is essential.

As shown in Table 2, DHPDH deficiency is characterized by the presence of abnormal amounts of uracil and thymine in urine [4] 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 DHU and DHT, and moderate amounts of uracil and thymine in urine [5]. Several methods reported for screening for disorders of pyrimidine metabolism are time-consuming or lack 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 [6]. GC–MS analysis of trimethylsilyl derivatives of urinary organic acid extracts could detect this deficiency [21] 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 [2]. Very recently a rapid and specific screening method for patients at risk of inherited disorders of pyrimidine and purine metabolism was described: it involves use of urine and HPLC–ESI-MS/MS [18], and targets uracil, thymine, 5-hydroxymethyluracil and orotate, but not DHT, DHU, creatinine or amino acids. Shoemaker and Elliott reported that after excessive urea in the urine is degraded with urease and removed, urinary organic acids, amino acids and sugars could be simultaneously analyzed using GC–MS [17]. Shoemaker's procedure, however, takes several hours for sample pretreatment and is not very practical. Based on our

Table 2
Different disorders of pyrimidine metabolism

Type	Uracil	Thymine	DHU	DHT	Orotate
DHPDH def.	↑↑	↑↑	–	–	–
DHP def.	↑	↑	↑↑	↑↑	–
Urea cycle disorders[a]	↑	–	–	–	↑
HHH syndrome[b]	↑	–	–	–	↑
LPI[c]	↑	–	–	–	↑
OCT carrier	↑	–	–	–	–
Orotic aciduria	–	–	–	–	↑↑
Benign persistent orotic aciduria	–	–	–	–	↑
Uraciluria	↑	–	–	–	–

[a] Urea cycle disorders except for deficiencies of *N*-acetylglutamate synthase and carbamoylphosphate synthase.
[b] Hyperornithinemia–hyperammonemia–homocitrullinuria syndrome.
[c] Lysinuric protein intolerance.

experiences with 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 [16]. Our rapid and simple pretreatment (1 h for one sample or 3 h for a batch of 30 samples), with the use of isotope dilution, and GC–MS measurement (15 min per sample) was also applied in a pilot study for neonatal mass screening [22,23]. This diagnostic procedure extended the chemical diagnostic capacity of gas chromatographic mass spectrometric urine metabolic profiling to new categories of metabolic disorders [24,25].

5FU toxicity also occurs in heterozygotes with DHPDH deficiency [10,20]. A frequency of putative heterozygotes of DHPDH deficiency as high as 3% was estimated based on catalytic activity levels in population studies [18] and this frequency allows the estimation of up to 1/1000 homozygotes for mutant DHPDH alleles [10]. Sumi et al. [3] estimated the prevalence of DHP deficiency to be approximately 1/10 000 births in Japan, as they found two asymptomatic cases of DHP deficiency among 20 000 infants examined by their HPLC method [19]. Although DHPDH carrier detection by analysis of enzyme activity in lymphocytes or fibroblasts has been reported to be unreliable, Gennip et al. suggested that such detection is possible if circadian rhythm is taken into consideration, and recommended collecting samples from suspected carriers, patients and non-carriers at the same time of the day [2]. As for DHP deficiency detection by analysis of urinary levels of DHU and DHT, Sumi et al. [3] found intra-and inter-assay variations to be small, indicating that DHP cases could be diagnosed through only one urine sampling. They also found that the heterozygotes showed slight increases of DHU and DHT, and that it was possible to distinguish them from DHP homozygotes, but it could not be judged whether individuals were heterozygous or normal, when urinary metabolites were analyzed by their HPLC method [3]. To differentiate between carriers and healthy controls, a uracil loading test was required; urinary DHU concentrations in the urine of carriers after uracil loading were several times higher than those in normal control persons [3]. Although it has been speculated that the risk of 5FU toxicity may be relatively low in DHP heterozygotes [3], we consider it possible that some heterozygotes may experience 5FU toxicity in the future. We therefore determined the urinary concentrations of pyrimidines in healthy newborns and subjects older than 4 years of age, as shown in Table 3, and their mean values and SD. We expressed these values relative to either creatinine concentration or creatinine plus creatine concentration.

The mass chromatograms of trimethylsilyl derivatives of metabolites from a urine sample in which uracil, thymine, DHU and DHT were spiked in different amounts simulating the elevated levels in typical and moderate cases and heterozygotes of DHPDH deficiency (Fig. 3 (1), (2) and (3), respectively, see also Table 1) and DHP deficiency (Fig. 4 (1), (2) and (3), respectively, see also Table 1) were

Table 3
Recovery, C.V. and control value of urinary pyrimidines[a]

	Recovery (%)	C.V. (%)	Control value mmol/mol Cre	
			neonate, $n=48$	>4Y, $n=27$
U	96	3.0	2.03±1.33	7.52±4.21
			(1.26±0.79)	(5.17±2.67)
T	104	3.9	0.56±0.27	0.29±0.16
			(0.40±0.24)	(0.19±0.08)
DHU	85	5.1	3.74±1.78	1.58±0.77
			(2.68±1.51)	(1.05±0.42)
DHT	89	5.4	2.87±1.31	0.83±0.38
			(2.06±1.13)	(0.57±0.27)
Orotate	83	2.7	2.49±1.29	0.66±0.30
			(1.59±0.91)	(0.45±0.20)

[a] (): per creatinine plus creatine.

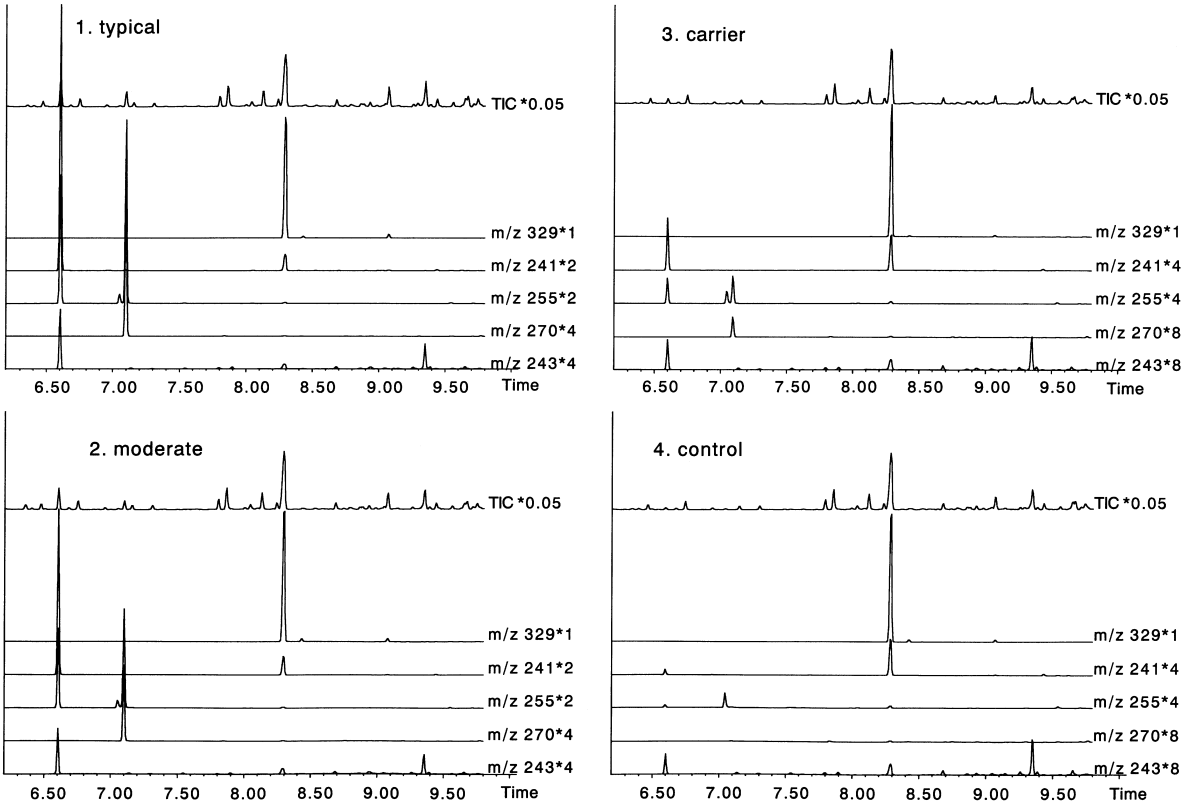

Fig. 3. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from artificially prepared urine specimens simulating dihydropyrimidine dehydrogenase deficiency (1: typical, 2: moderate, 3: carrier) or a control (4). Except for creatinine-3, mass chromatogram intensity in 3 and 4 is expressed as twice as that in 1 and 2 in order to clearly show the difference between control and carrier. The ions targeted were m/z 329 for creatinine, m/z 243 for $^{15}N_2$-uracil (IS), m/z 241 for uracil and m/z 255 and 270 for thymine.

obtained. The findings indicated that it should be easy and rapid to make a rapid differential diagnosis by simultaneously analyzing pyrimidine derivatives and creatinine with the use of stable isotope-containing internal standards. Heterozygote detection, however, appeared to be difficult using artificial urine specimens. A TIC chromatogram for typical DHP deficiency (see Fig. 4(3)) is shown in Fig. 5.

No patients with deficiency of UP have been described yet. Patients with β-alanine α-ketoglutarate aminotransferase (BAKAT) deficiency [26] and patients with proven partial BAKAT deficiency [27] both showed persistently elevated urinary β-alanine levels. Hyper-β-aminoisobutyric aciduria due to β-aminoisobutyrate pyruvate aminotransferase (BAIBPAT) deficiency is thought to be due to a benign polymorphism [28]. In our procedure, amino acids, including ω-amino acids such as β-alanine, β-aminoisobutyrate, γ-aminobutyrate and glycine, are also recovered and simultaneously analyzed, although trimethylsilylation of each ω-amino acid yields two peaks corresponding to the di- and tri-trimethylsilyl derivatives [25,29]. ω-Amino acids di-TMS and tri-TMS were determined using d_2-glycine di-TMS and tri-TMS respectively as an internal standard in our procedure [16]. The procedure can also be applied for simultaneous quantitation of uracil and orotate, which is essential for the detection of urea cycle disorders except for carbamoylphosphate synthase deficiency and *N*-acetylglutamate synthase deficiency, hyperornithinemia–hyperammonemia–homocitrullinuria syndrome and lysinuric protein intolerance (Table 2). In DHPDH deficiency, DHT, DHU and orotate are within the normal range,

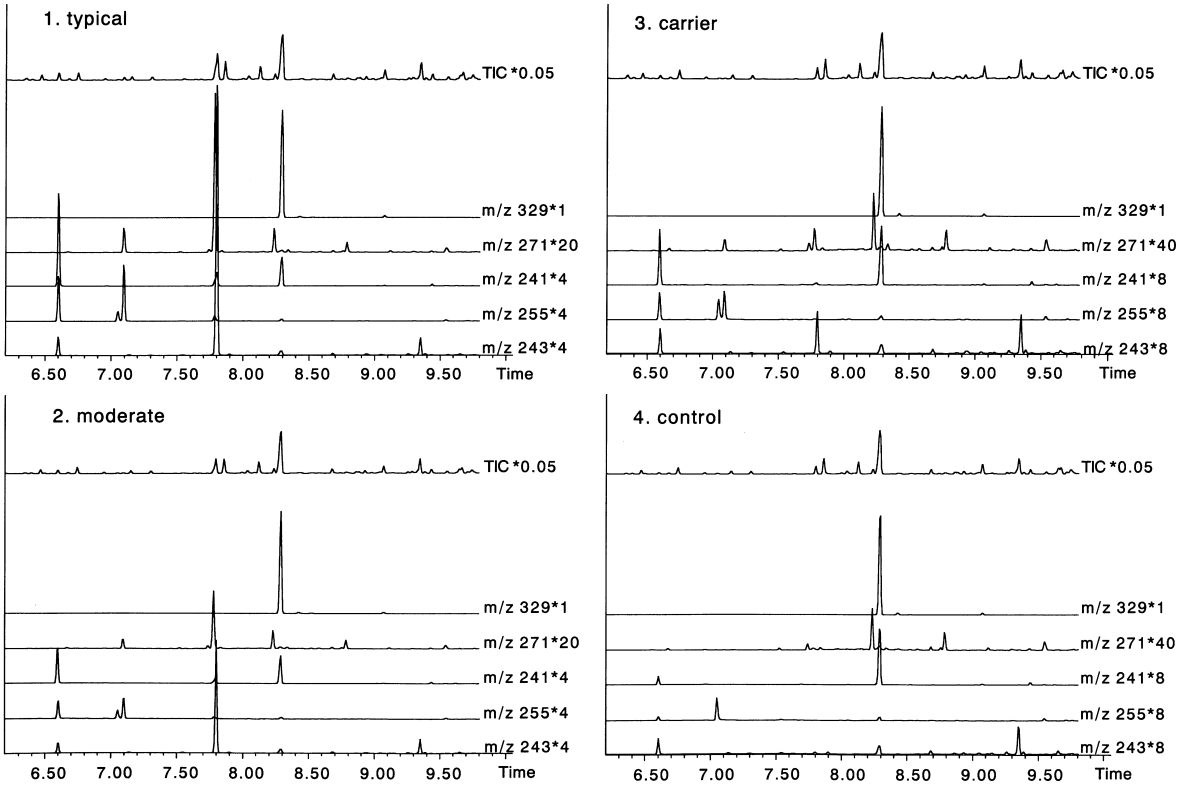

Fig. 4. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from artificially prepared urine specimens simulating dihydropyrimidinase deficiency (1: typical, 2: moderate, 3: carrier) or a control (4). Except for creatinine-3, mass chromatogram intensity in 3 and 4 is expressed as twice as that in 1 and 2 in order to clearly show the difference between control and carrier. The ions targeted were m/z 329 for creatinine, m/z 243 for $^{15}N_2$-uracil (IS), m/z 241 for uracil, m/z 255 for thymine, m/z 271 for 5, 6-dihydrothymine and m/z 243 for 5, 6-dihydrouracil.

and in DHP deficiency, DHT and DHU are significantly increased, and uracil and thymine moderately increased, while orotate is within the normal range. As urinary uracil is also increased in ornithine carbamoyltransferase (OCT) carriers [30], simultaneous determination of thymine and uracil is desirable. In heritable orotic aciduria, only orotate is increased (Table 2). We have demonstrated that in our procedure orotate is a valuable index for evaluation of folate deficiency, which causes impaired DNA synthesis, megaloblastic anemia, enhanced pyrimidine biosynthesis and orotic aciduria [25]. The recovery of orotate (83%) in our simplified urease treatment procedure was the lowest among the pyrimidines examined in the present study, but was markedly higher than that obtained by conventional solvent extraction; 31% with ethyl acetate twice with sodium chloride under acidic conditions [31].

The present study has shown that simplified urease treatment enables simultaneous and quantitative determination of thymine, uracil, DHT, DHU, orotate and creatinine in 0.01–0.1-ml liquid urine samples or urine-soaked filter paper strips, with a procedure involving urease treatment, isotope dilution and GC–MS. This simple, rapid and highly sensitive diagnostic procedure can be highly recommended for differential diagnosis of homozygous patients and heterozygotes in the pyrimidine degradative pathway, especially when considering the administration of pyrimidine analogues such as 5FU.

Acknowledgements

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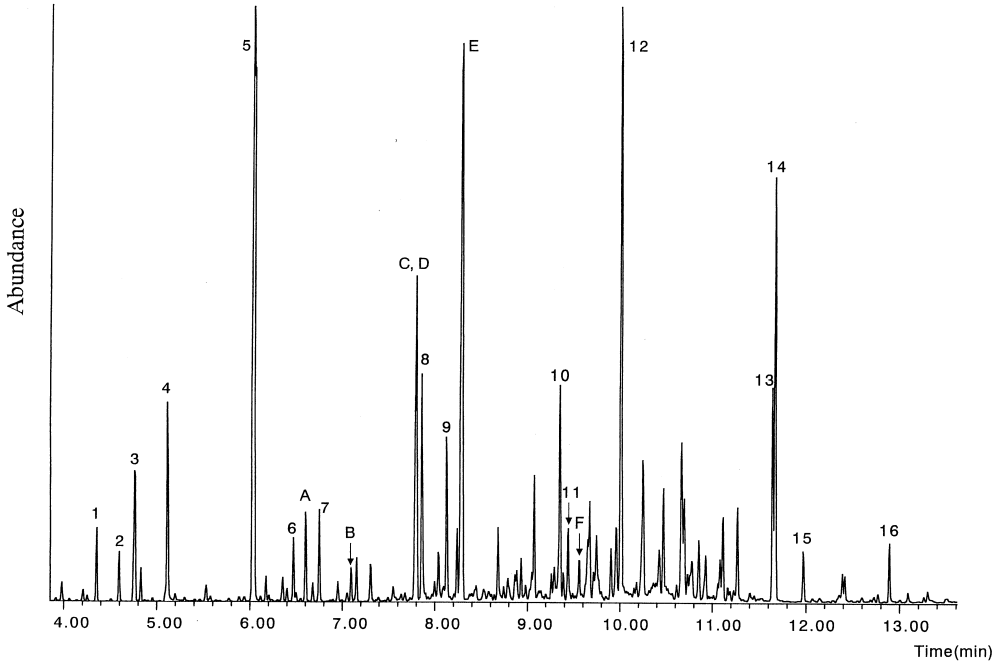

Fig. 5. TIC chromatogram of trimethylsilyl derivatives of metabolites from artificially prepared urine specimens simulating dihydropyrimidinase (DHP) deficiency (1: typical). Peak identifications are: (1) glycolate; (2) alanine; (3) glycine; (4) sulfate; (5) phosphate and leucine; (6) 2, 2-dimethylsuccinate (spiked); (A) uracil and $^{15}N_2$-uracil; (7) serine; (B) thymine; (C, D) 5, 6-dihydrothymine and 5, 6-dihydrouracil; (8) erythritol; (9) tetronate; (E) creatinine and D_3-creatinine; (10) xylitol; (11) 2-hydroxyundecanoate (spiked); (F) orotate and $^{15}N_2$-orotate; (12) citrate; (13) myo-inositol; (14) urate; (15) *n*-heptadecanoate (spiked); (16) pseudouridine.

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