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Rapid gas chromatographic–mass spectrometric diagnosis of dihydropyrimidine dehydrogenase deficiency and dihydropyrimidinase deficiency

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Abstract

A rapid yet reliable chemical diagnosis for dihydropyrimidine dehydrogenase (DHPD) deficiency, and possibly dihydropyrimidinase (DHP) deficiency in cancer patients, prior to therapy with pyrimidine analogues such as 5-fluorouracil, is desired for prevention of severe side-effects by these drugs. We have reported the basic separation and quantitation technology for pyrimidine metabolites using gas chromatography–mass spectrometry. A proposal to use the number (*n*) of standard deviations (SD) above the normal mean, as the index of the excessive urinary excretion of the metabolites appears not to be commonly used. When used, the values were too small, such as two or three, even in genetic disorders. Here, we applied the method to 11 urine specimens from proven cases including two DHP carriers and proved how specific the method is, because ''*n*''-values were markedly large for thymine (T), uracil (U) and/or dihydrothymine (DHT) and dihydrouracil (DHU). In three cases with DHPD deficiency, two were siblings, one with symptoms and the other without, *n* was 12 for T and 5.9 for U, and 5-hydroxymethyluracil was distinctly detected. These values indicate that the nature of genetic mutation relates closely to the degree of metabolite accumulation in pyrimidine disorders. In six patients with DHP deficiency, *n* was 8.4–12 for DHT and 7.2–11 for DHU. Many mutations are known for both genes and the assay of residual enzyme activity may be time-consuming or invasive especially for those with DHP deficiency. Thus, this noninvasive yet comprehensive urinalysis has great value for those without a family history, as the first trial, before DNA or the enzyme assay. Our findings again raise the question whether the metabolic block really causes the symptoms found in pyrimidine disorders.

Keywords: Enzymes; Dihydropyrimidine dehydrogenase; Dihydropyrimidinase

1. Introduction

In humans, pyrimidines are degraded in four steps that are catalyzed by dihydropyrimidine dehydro-

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genase (DHPD, EC 1.3.1.2), dihydropyrimidinase (DHP, EC 3.5.2.2, liver-specific), β-ureidopropionase (UP, EC 3.5.1.6), and three aminotransferases. The clinical presentations in patients with DHPD deficiency (MIM 274270) and DHP deficiency (MIM 222748) are variable, and many asymptomatic cases have been reported [1–3]. Many mutations are known for both genes and the assay of residual enzyme activity may be time-consuming or invasive especially for DHP. Because pyrimidine analogues such as 5-fluorouracil (5FU) are metabolized by these enzymes [4], cancer patients with DHPD deficiency suffer from severe neurotoxicity, sometimes leading to death, following therapy with these drugs [5,6]. Patients with DHP deficiency are also possibly at risk [3,7]. We have reported the diagnostic procedure that involves stable isotope dilution, urease-pretreatment, and gas chromatography–mass spectrometry (GC–MS) [8]. The recoveries of the indicators, uracil (U), thymine (T), 5,6-dihydrothymine (DHT), 5,6-dihydrouracil (DHU), and 5-hydroxymethyluracil (HMU), were high due to no fractionation [9,10]. Here, we applied the method to 11 urine specimens from proven DHPD and DHP cases including two DHP carriers. A proposal to use the number (*n*) of standard deviations (SD) above the normal mean, as the index of excessive urinary excretion of the metabolites, appears not to be commonly used. We evaluated our method from the viewpoint of its sensitivity and specificity using the above index (*n*) values. We also examined in the DHPD-deficient siblings, one with symptoms and the other without, and in asymptomatic DHP deficient siblings, whether the nature of genetic mutation relates closely to the value *n*, in pyrimidine degradation disorders.

2. Subjects

Urine specimens from 11 patients were examined: three patients had DHPD deficiency, six had DHP deficiency, and two were heterozygous for DHP. The patients' age when the urine specimens used for the present study were collected, the presence or absence of symptoms, the age of onset of symptoms, and the mutation are summarized in Table 1. Some information on Japanese DHP cases was published previously [3].

3. Experimental

3.1. Chemicals

1,3-$^{15}N_2$-U 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 98%, as judged by the lack of additional peaks on GC–MS. U, T, DHT, DHU, HMU, and urease type C-3 were obtained from Sigma (St Louis, MO, USA).

Table 1
Characteristics of patients with DHPD deficiency or DHP deficiency and DHP carriers

Case	Age at sampling	Age of onset, presence or absence of symptoms, mutation
DHPD1	2Y	6Y
DHPD2	4Y	No symptoms, sibling of symptomatic DHPD3
DHPD3	7Y	6Y, sibling of DHPD2
DHP1	6M	No symptoms, also has tyrosinemia
DHP2	7M	No symptoms, detected by screening, T68R/R490T[a]
DHP3	1Y7M	No symptoms
DHP4	9Y	8Y
DHP5	37Y	No symptoms, Q344R[a]
DHP6	38Y	No symptoms, sibling of DHP5, Q344R[a]
DHP7	20Y	No symptoms, heterozygote, mother of DHP2, R490T[a]
DHP8	20Y	No symptoms, heterozygote, father of DHP2, T68R[a]

Y, years; M, months.
[a] Patients previously described with their mutations [3].

3.2. Sample preparation, GC–MS analysis and quantification

Less than 0.1 ml of liquid urine or an eluate from a urine-soaked filter paper strip was used. For quantification, 4, 4, and 100 nmol of stable-isotope-labeled U, orotate, and creatinine, respectively, were spiked into 0.1 ml of urine and used as internal standards. The labeled U was used as the internal standard for U, T, DHT, and DHU, the labeled orotate was for orotate and HMU, and the labeled creatinine for creatinine [8]. The procedures for sample preparation, urease pretreatment, and GC–MS measurement including the speed and mass range for scanning, were exactly as described previously [9,10]. Aliquots (0.5 or 1 μl) of derivatized extracts were injected into a bench-top Hewlett-Packard GC–MSD (HP6890/MSD5973) using an automatic injection mode with a split ratio of 1:10–1:30. Because this method yields a total value for creatinine plus creatine, creatinine and creatine were also measured separately using an autoanalyzer (CX5, Beckman), and the urinary metabolite levels were evaluated on the basis of both standards: creatinine, and creatinine plus creatine.

The mass spectra of target compounds (U, T, DHU, DHT, orotate and creatinine) and internal standards ($^{15}N_2$-U, $^{15}N_2$-orotate and d_3-creatinine), ions used for quantitation, recovery, calibration curves, and intra-assay variability have been reported [8]. Mass chromatographic quantification of metabolites in urine samples was the same as described previously [8]. The ions used for quantification of HMU were m/z 358 and m/z 343.

4. Results

The total ion current (TIC) chromatograms of metabolites in the urine of the patients are shown in Fig. 1. Markedly increased excretion of U, T, and HMU was observed in DHPD-1 (a). Greatly increased excretion of DHU, DHT, and T as well as moderately increased U, was shown in DHP-deficiency (b). Mass chromatograms for three DHPD-deficiency cases and the control, and for three DHP-deficiency cases and the control are shown in Fig. 2 (1–4) and Fig. 3 (1–4), respectively. In urine from patient DHP1, *p*-hydroxyphenyllactate, phenylalanine, tyrosine, *p*-hydroxyphenylacetate, and *N*-acetyltyrosine were also increased (Fig. 1b). These compounds are associated with the catabolism of aromatic amino acids, indicating that DHP1 also had an inborn error of tyrosine metabolism. At the time of this investigation, patient DHP1, whose diagnosis was tyrosinemia type II, had no liver pathology, nor any eye or skin abnormalities.

The urinary concentrations of pyrimidines and dihydropyrimidines in DHPD deficiency and DHP deficiency were quantified (Tables 2 and 3). In the DHPD-deficient cases, the concentrations of U were greater than 299 mmol/mol creatinine, and the concentrations of T were greater than 118 mmol/mol creatinine, whereas DHU and DHT were undetectable. The concentrations of DHU and DHT in six DHP-deficient cases were greater than 101 mmol/mol creatinine and 78 mmol/mol creatinine. Neither β-ureidopropionate nor β-ureidoisobutyrate, the levels of which increase in patients with βUP deficiency, were detected in any of the samples examined.

The frequency distributions of the concentrations of U, T, DHU, DHT, and orotate in healthy controls were not normally distributed, but were skewed to the left. Consequently, we carried out a $\log_{10}$ transformation of the data before performing the statistical analysis. We used here the number (n) of standard deviations (SD) above the mean for healthy control, as the index of the excessive urinary excretion of the metabolites (the level in a patient = mean level in age-matched healthy control + $n \times$ SD). For example, the value n for U in DHPD-1 was defined as 5.9, as the concentration of U was 5.9 SD above the mean. In Table 4, the abnormality n, is shown for all the samples examined in the present study.

5. Discussion

The clinical presentation of deficiencies of DHPD and DHP is variable, not dependent on the nature of mutation, and many asymptomatic cases have been described [1–3,7,11]. Even asymptomatic homozygotes and heterozygotes for DHPD suffer from severe side-effects from pyrimidine analogues [5,6]. Such side-effects are likely to occur for those with

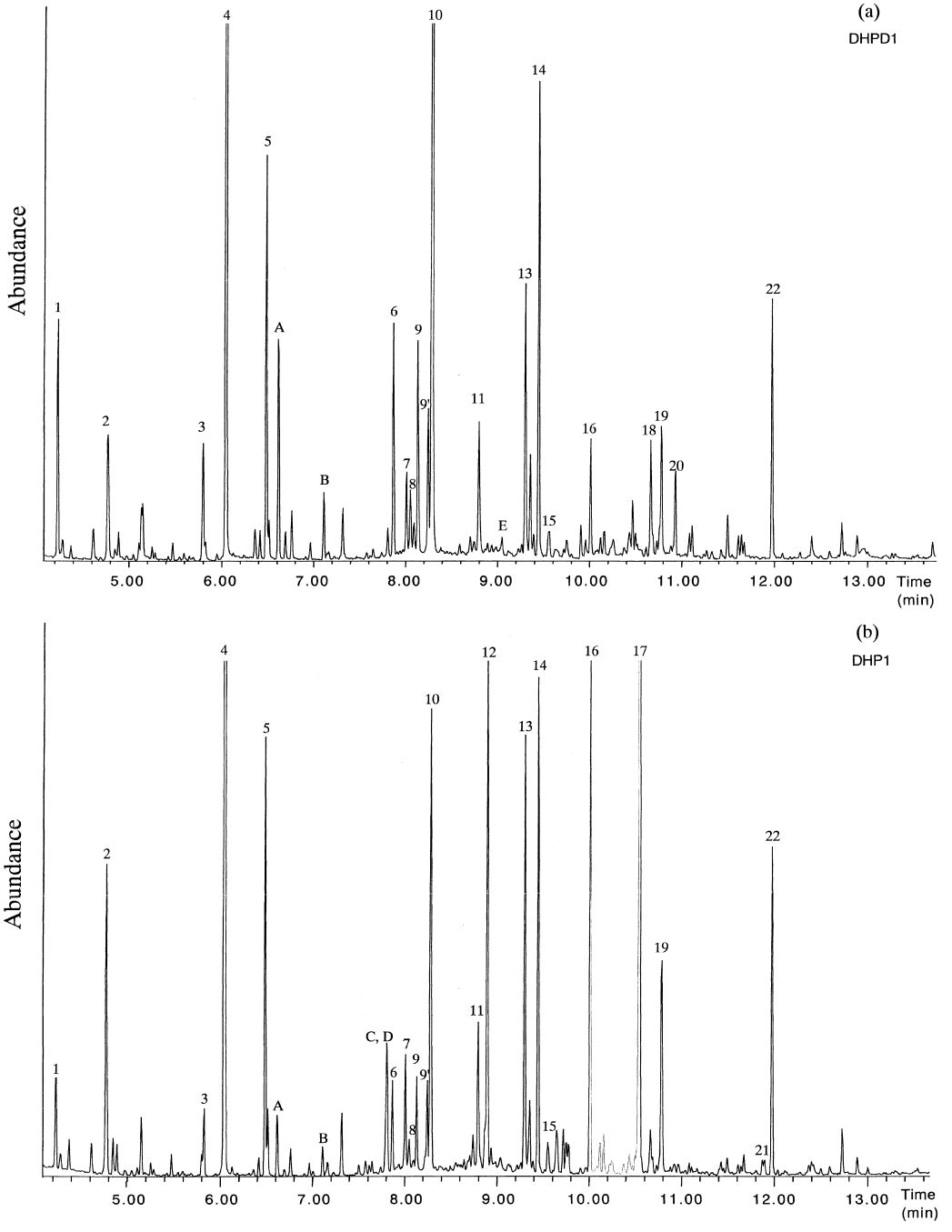

Fig. 1. TIC chromatograms of trimethylsilyl derivatives of metabolites from urine of DHPD1 (a), and DHP1 (b). Peaks are: (1) lactate-2; (2) d_0- and d_5-glycine-2; (3) urea-2; (4) phosphate-3; (5) 2,2-dimethylsuccinate (IS_1); (A) $^{15}N_0$- and $^{15}N_2$-U-2; (B) T-2; (C) DHT; (D) DHU; (6) erythritol-4; (7) d_0- and d_3-methionine-2; (8) 5-oxoproline-2; (9 and 9′) threonate-4 and erythronate-4; (10) d_0- and d_3-creatinine-3; (11) d_0- and d_5-phenylalanine-2; (12) 4-hydroxyphenylacetate-2; (E) HMU-3; (13) d_0- and d_4-lysine-3; (14) 2-hydroxyundecanoate-2 (IS_2); (15) $^{15}N_0$- and $^{15}N_2$-orotate-3; (16) citrate-4; (17) 4-hydroxyphenyllactate-3; (18) d_0- and d_4-lysine-4; (19) d_0- and d_4-tyrosine-3; (20) glucose-5; (21) *N*-acetyltyrosine-2; (22) heptadecanoate-1 (IS_3).

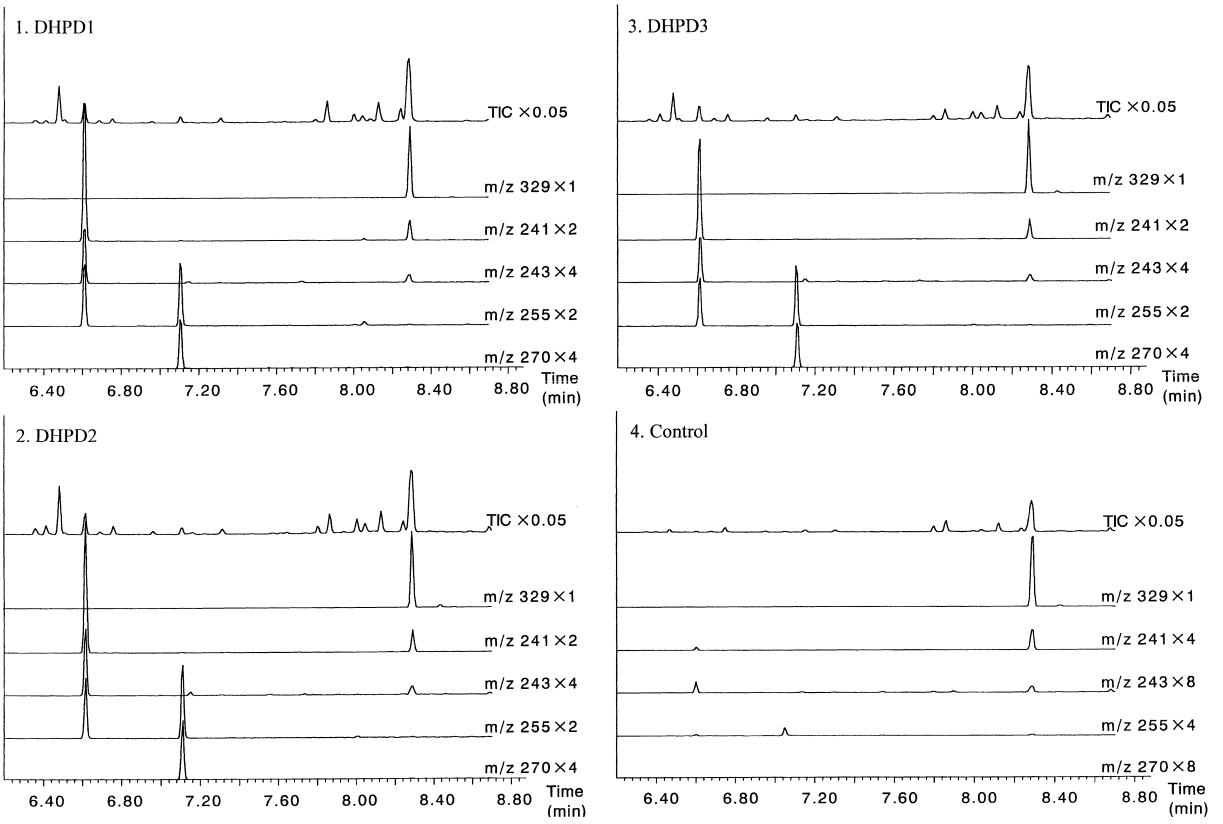

Fig. 2. Mass chromatograms of trimethylsilyl derivatives of metabolites from the urine of patients with DHPD deficiency (1, DHPD1; 2, DHPD2; 3, DHPD3) and a control (4). To clearly show the difference between the results for the control and the patients, the mass chromatogram intensity in 4 is expressed as twice that in 1–3, except for creatinine-3. The ions targeted were $[M]^+$ at m/z 329 for creatinine-3, $[M\text{-}15]^+$ at m/z 243 for $^{15}N_2$-U-2 (IS) and m/z 241 for U-2, and $[M\text{-}15]^+$ at m/z 255 and $[M]^+$ at m/z 270, for T-2. The ion of m/z 255 due to $[M\text{-}H]^+$ was also seen at U-2.

DHP deficiency as well [3]. Therefore, it is critical to identify cancer patients who should not be treated with these drugs. High-pressure liquid chromatography (HPLC) [12,13], amino acid analysis [14], and nuclear magnetic resonance spectroscopy [15] have been used for these purposes. These methods, however, had difficulty in quantitation, lacked sensitivity or specificity, or required further analysis to establish a differential diagnosis. Very recently, a rapid screening method for deficiencies of DHPD, DHP, and UP has been described; it uses HPLC–ESI-MS–MS and targets U, T, DHT, DHU, β-ureidopropionate, and β-ureidoisobutyrate [16]. GC–MS is more popular than HPLC–ESI-MS–MS. The conventional GC–MS analysis of urinary organic acid extracts, however, is not as sensitive as two-dimensional thin-layer chromatography or HPLC [2,17] and quantification was not possible because of variable extraction yields [2]. Urease-pretreatment without fractionation and with isotope dilution permitted the GC–MS procedure to be used for quantitative pyrimidine analysis [8]. The procedure also permitted GC–MS to be used to detect individuals with UP deficiency [18], which was impossible with conventional solvent extraction, because of the chemical nature of β-ureides.

Although it is a common idea to use the number (n) of standard deviations (SD) above the normal mean as the index of the excessive urinary excretion of metabolites, the index appears not to be commonly used. When used, the values were too small even in genetic disorders. The reason is probably the low

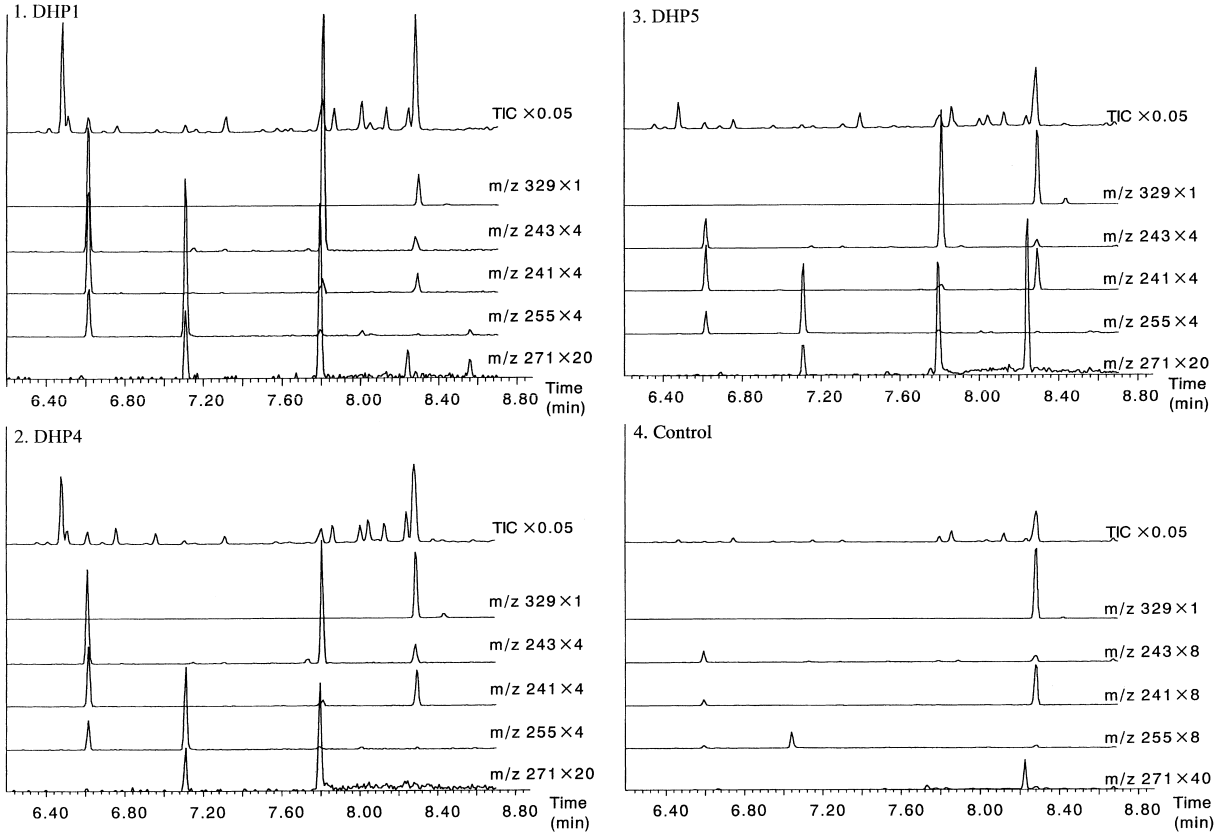

Fig. 3. Mass chromatograms of trimethylsilyl derivatives of metabolites from the urine of patients with DHP deficiency (1, DHP1; 2, DHP4; 3, DHP5) and a control (4). To clearly show the difference between the control and the patients, the mass chromatogram intensity in 4 is expressed as twice that in 1–3, except for creatinine-3. The ions targeted were m/z 329 for creatinine-3, m/z 243 for $^{15}N_2$-U-2 (IS), m/z 241 for U-2, m/z 255 for T-2, m/z 271 for DHT, $[M\text{-}H]^+$, and m/z 243 for 5, 6-DHU-2, $[M\text{-}15]^+$. The ion of m/z 271, due to the isotope of $[M],^+$ was also seen at T-2.

Table 2
Urinary concentrations of pyrimidines and dihydropyrimidines in three cases with DHPD deficiency

Case	U	T	DHU	DHT	HMU
DHPD1	576 (379)	180 (119)	UD	UD	69.0 (45.4)
DHPD2	299 (196)	120 (78.8)	UD	UD	28.2 (18.5)
DHPD3	302 (200)	118 (78.0)	UD	UD	26.9 (17.8)
Control (mean±1SD)					
Newborn	1.62–3.57	0.31–0.87	1.56–6.75	1.59–4.52	UD
>4 years	4.44–15.1	0.15–0.42	0.79–2.45	0.49–1.18	UD

The values are expressed as mmol/mol creatinine and mmol/mol creatinine plus creatine in parentheses. UD, undetectable.

Table 3
Urinary concentrations of pyrimidines and dihydropyrimidines in six cases with DHP deficiency and two DHP carriers

Case	U	T	DHU	DHT
DHP1	335	334	857	675
	(192)	(191)	(490)	(386)
DHP2	253	190	556	456
	(89.0)	(66.9)	(196)	(161)
DHP3	182	171	537	438
	(142)	(134)	(420)	(343)
DHP4	40.7	38.1	101	78.3
	(37.8)	(35.4)	(94.1)	(72.7)
DHP5	35.5	40.8	132	106
	(33.8)	(38.9)	(126)	(101)
DHP6	36.7	26.8	103.1	81.5
	(33.5)	(24.5)	(94.2)	(74.5)
DHP7	5.90	0.370	1.38	0.589
	(5.19)	(0.326)	(1.21)	(0.518)
DHP8	9.13	0.558	1.62	1.54
	(8.86)	(0.542)	(1.57)	(1.50)
Control (mean±1SD)				
Newborn	1.62–3.57	0.31–0.87	1.56–6.75	1.59–4.52
>4 years	4.44–15.1	0.15–0.42	0.79–2.45	0.49–1.18

The values are expressed as mmol/mol creatinine and mmol/mol creatinine plus creatine in parentheses. DHP7 and DHP8 are heterozygotes.

specificity or sensitivity of methods that had been employed for patients and age-matched controls. In urine from patients with DHPD deficiency, *n* was 5.9, even for the target U and 12 for the target T, which deviated more-markedly from the control than U, when creatinine was used as the standard. The *n*-value was 5.5–5.7 for U and 11–15 for T, respectively, when creatinine plus creatine (total creatinine) was used as the standard. For patient DHPD1, whose age at disease onset was 6 years, the urine examined was taken at 2 years, when the child was presymptomatic. DHPD2, who was asymptomatic, and DHPD3, who had developed symptoms, were siblings. The results from the present study suggest that the abnormalities in the metabolite level reflect the nature of the genetic abnormality but do not relate to the presence or absence of clinical abnormalities.

In DHP-deficient patients, the *n*-values for DHU, DHT, and T were 7.2–11, 8.4–12, and 8.7–12, respectively, when creatinine was used as the standard, and 6.1–10, 6.8–13, and 10–13, respectively, when total creatinine was used as the standard. U also increased moderately. The sample from patient DHP2, whose mutation was T68R/R490T, showed *n*-values for DHU, DHT, and T that were all above 7.2, while the *n* for orotate was 3. The value of *n* was not the highest for DHP4, whose urine was collected 1 year after disease onset, and was the only symptomatic patient with DHP. These results suggest that the value of *n* does not depend on the presence or absence of clinical symptoms. The *n* for T in DHP-deficient patients was greater than for U and was on the same order as observed for DHU and DHT. This finding may suggest that the metabolic pathway for U is more diverse than the pathway for T. In DHP5 and DHP6, who are siblings and homozygous for Q344R [3], the metabolic profiles were very similar and the *n*-values for U, T, DHU, and DHT differed only slightly, by 4, 11, 8, and 10%, respectively. U may have been a more quantitative indicator than T, DHU, or DHT, because stable isotope-labeled U was used as the internal standard for all four of these targets.

Because of their risk for side-effects from pyrimidine analogues, it is desirable to identify DHPD carriers [2]. We could not examine if the method could detect DHPD carriers, because no urine samples from DHPD carriers were available. Sumi et al. suggested that a U loading test is necessary to detect DHP carriers in their method [3]. We also found it impossible to detect DHP carriers in our GC–MS procedure, as DHP7 and DHP8 (the parents of DHP2) did not show any abnormality. It is, however, suggested that DHP carriers are not at risk for the severe side-effects from pyrimidine analogues [3].

Urine is preferable to blood for screening for pyrimidine degradation disorders [2]. We could prove the specificity of the GC–MS method using less than 0.1 ml of urine within 1.5 h. No further analysis is required to make a differential diagnosis because secondary pyrimidinuria and dihydropyrimidinuria can be differentiated by simultaneous quantification of methylcitrate, methylmalonate, creatinine and orotate [19]. The present findings again raise the question whether the real cause of the symptoms found in pyrimidine disorders is the metabolic block. Many mutations are known for both genes and the assay of residual enzyme activity may be time-consuming or invasive especially for DHP, which is expressed only in the liver. Thus, this noninvasive

Table 4
Abnormality n in the level above mean$+n\times$SD in 11 cases examined in the present study

Case	Age	Control	U	T	DHU	DHT	Orotate
DHPD1	2Y	1–4Y	5.9	12	UD	UD	0.8
			(5.5)	(11)			(0.9)
DHPD2	4Y	>4Y	5.9	12	UD	UD	0.8
			(5.7)	(15)			(0.5)
DHPD3	7Y	>4Y	5.9	12	UD	UD	1.2
			(5.7)	(15)			(1.0)
DHP1	6M	1–12M	4.1	10	7.8	8.9	2.5
			(4.5)	(12)	(7.3)	(8.0)	(2.1)
DHP2	7M	1–12M	3.7	9.4	7.2	8.4	4.8
			(3.4)	(10)	(6.1)	(6.8)	(3.3)
DHP3	1Y7M	1–4Y	4.2	12	11	12	2.9
			(4.2)	(12)	(9.1)	(10)	(3.2)
DHP4	9Y	>4Y	2.6	9.6	7.6	10	−3.0
			(3.0)	(13)	(9.7)	(13)	(−2.5)
DHP5	37Y	>4Y	2.4	9.7	8.0	11	0.9
			(2.8)	(13)	(10)	(13)	(1.5)
DHP6	38Y	>4Y	2.3	8.7	7.4	10	3.2
			(2.8)	(12)	(9.7)	(13)	(4.0)
DHP7	ca. 20Y	>4Y	−0.5	0.8	−0.02	−0.6	−0.1
			(−0.2)	(1.5)	(0.5)	(−0.03)	(0.3)
DHP8	ca. 20Y	>4Y	0.2	1.5	0.3	1.6	0.2
			(0.8)	(2.9)	(1.2)	(2.9)	(1.0)

Values are expressed based on creatinine while those in parentheses are based on total creatinine. UD, undetectable; DHP7 and DHP8 are heterozygotes; Y, years; M, months.

yet highly specific urinalysis has great value for those without a family history, as the first trial.

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