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Journal of Chromatography B, 746 (2000) 75–82

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Rapid, simplified and sensitive method for screening fructose-1,6-diphosphatase deficiency by analyzing urinary metabolites in urease/direct preparations and gas chromatography–mass spectrometry in the selected-ion monitoring mode

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Abstract

Children with fructose-1,6-diphosphatase (FDPase) deficiency often experience life threatening episodes such as ketotic hypoglycemia. We report here a rapid, simplified and sensitive method to analyze glycerol-3-phosphate (G3P) and glycerol in urine, that can be used to detect FDPase deficiency. We used the urease/direct preparation and gas chromatography–mass spectrometry in the selected-ion monitoring mode, enabling detection of G3P and glycerol level in normal controls. Using this approach, FDPase deficiency can be more easily diagnosed and differentiated from glycerol kinase deficiency or glycerol infusion patients. To date, diagnosis has been essentially based on the assay of enzymes in the liver. The proposed non-invasive method provides a clinically significant diagnostic tool that may help prevent episodic attacks.

Keywords: Enzymes; Fructose-1,6-diphosphatase

1. Introduction

Fructose-1,6-diphosphatase (FDPase) deficiency, first described by Baker and Winegrad in 1970 [1], is an autosomal recessive inherited metabolic disorder of gluconeogenesis clinically characterized by episodes of hyperventilation, apneic spells, hypotonia, metabolic acidosis or unconsciousness probably the result of hypoglycemia [2]. The episodes often begin during the neonatal period or early infancy, and might be life-threatening, even mimicking Reye's syndrome [3]. Although these attacks can be promptly overcome by infusion of glucose and bicarbonate, a considerable number of patients could die of 'unknown origin' if action is not taken. With early detection and adequate management, patients with FDPase deficiency could achieve normal growth and development and attacks could be prevented.

Increased excretion of glycerol or sugar phosphates, including glycerol-3-phosphate (G3P) during acute crisis has been reported [4–7]. These metabolites were measured by GC–MS with extraction,

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00123-7

using a DEAE Sephadex column (column method). If detection or screening of this disorder by GC–MS analysis is feasible, it would be of clinical significance for early detection, thus preventing episodic attacks and their debilitating effects.

Matsumoto and Kuhara described a rapid, simplified screening method using GC–MS and urease treatment, direct evaporation and a trimethylsilylation preparation (urease/direct method) [8], which enables simultaneous analysis of organic acids, amino acids, sugars and alcohols. We have since determined the sensitivity, reproducibility and practicability of a modified method using the urease/direct method and GC–MS in the SIM mode (GC–MS-SIM), and compared our findings with those obtained with the column method. We analyzed metabolites, including G3P or glycerol, in urine from patients with FDPase deficiency during the acute phase and stable periods as well as in patients with glycerol kinase (GK) deficiency and patients undergoing glycerol infusion treatment.

2. Materials and methods

2.1. Reagents

G3P and urease of Sigma type III from Jack beans (31000 units/g solid) were purchased from Sigma (St. Louis, MO, USA). Tropic acid (TA), margaric acid (MGA) and *n*-pentadeconoic acid (PDA) were purchased from Nakalai Tesque (Kyoto, Japan). *N,O*-Bis (trimethylsilyl) trifluoacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained from GL Science (Tokyo, Japan). DEAE Sephadex A25 powder was obtained from Pharmacia Biotech (Uppsala, Sweden).

2.2. Urease/direct method

The sample preparation was carried out according to the method described by Matsumoto and Kuhara [8] with some modifications. Briefly, a urine specimen containing 0.1 mg of creatinine was incubated with 30 U of urease at 37°C for 30 min to degrade the urea. A 1-ml volume of ethanol was added to the urine sample to precipitate protein, then the ethanol and urine mixture was centrifuged at 1000 *g* for 5 min. The supernatant was transferred to a 4.0-ml vial tube and 20 μg each of TA and MGA were added as internal standards (I.S.s). The aliquot was gently dried under a stream of nitrogen at 50°C. The dry residue was trimethylsilyl (TMS)-derivatized with 100 μl of BSTFA–TMCS (10:1, v/v) for 30 min at 80°C.

2.3. Column method

Preparations were made as described previously [7] with some modifications. To urine specimens containing 0.1 mg of creatinine, 20 μg each of TA and MGA were added as I.S.s. To precipitate inorganic phosphate and sulfate, 1.0 ml of saturated barium hydroxide solution was added. After centrifugation at 1000 *g* for 5 min, the supernatant was neutralized by adding drops of 1 *M* HCl and was then applied to a DEAE column (4 cm×7 mm I.D.). After washing with distilled water, the organic acid fraction was eluted with 4.0 ml of 1.5 *M* of pyridinium acetate buffer (pH 6.0). The eluent was then dried under a stream of nitrogen gas at 50°C and the dry residue was TMS-derivatized, similar to the urease/direct method.

2.4. GC–MS analysis

The GC–MS equipment used was Shimadzu GCMS QP5000 (Kyoto, Japan). The column used was 30 m×0.25 mm I.D., coated with 5% phenylmethyl silicone with a 0.25-μm film thickness (DB-5, J&B Scientific, Fulsom, CA, USA). The temperature program was started at 100°C with an initial holding of 1 min and was raised at a rate of 10°C/min to 290°C with a final holding of 10 min. The injection port and transfer line were both maintained at 280°C.

A 1-μl volume of the TMS-derivatized sample was injected into the GC–MS system and analyzed by split mode with a split ratio of 1:10. The flow-rate of the helium carrier gas was ≈1.5 ml/min and the linear velocity was 40.2 cm/s. SIM was carried out using a mass selective detector equipped with an electron impact ion source. Table 1 shows the selected ions, time windows, estimated retention times and methylene unit (MU) values for each compound. We chose a selected ion-1 for quantifica-

Table 1
Selected ions, time windows in GC–SIM-MS analysis, and MU values

Compound	Selected ion 1 (Q-ion)	Selected ion 2 (C-ion)	Time window (min)	Retention time estimated (min)	(MU)
Target compound					
Lactic acid	219	190	3.0–8.0	3.57	(10.45)
3-Hydroxybutyric acid	233	191	3.0–8.0	4.53	(10.38)
Glycerol	218	205	3.0–8.0	6.16	(12.78)
Glycerol-3-phosphate	357	299	11.0–15.0	12.34	(17.81)
Internal standard					
Tropic acid	280	267	8.0–11.0	10.23	(15.99)
n-Pentadecanoic acid	299	314	11.0–15.0	14.06	(19.42)
Margaric acid	327	145	15.0–18.0	16.03	(21.41)

tion (Q-ion) and selected ion-2 for conformation (C-ion). The retention time for each compound was calculated from analysis of the hydrocarbon mixture (C_{10}–C_{26}) and each MU value. The same samples were also analyzed separately by the scan mode in order to identify the compounds according to their mass spectra.

2.5. Comparison of recovery and variation of quantification between urease/direct method and column method

A standard solution containing 50 nmol/ml each of lactic acid (LA), 3-OH-butyric acid (3HB), and G3P, and 25 nmol/ml of glycerol was prepared. A 1-ml portion of the solution was treated by either the urease/direct or the column method, and then analyzed using GC–SIM-MS. For comparison of recovery, 20 μg of PDA were added before the TMS-derivatization, and peak relative area (PRA, %) of Q-ion for each component to that of PDA (*m*/*z* 299) in the SIM was calculated. Quantification was carried out from the PRA (%) of the Q-ions of each compound to the that of the I.S. (TA, *m*/*z* 280).

2.6. Patients

The clinical and biochemical profiles of four Japanese patients with FDPase deficiency are summarized in Table 2. The diagnosis for these patients

Table 2
Clinical profiles of four patients with FDPase deficiency

Case (age in years, gender)	Age at onset	Clinical findings at onset	Age at diagnosis (years)	Clinical findings at diagnosis	Enzyme activity (tissue tested)
1 (2, M)	2 d	Vomiting Tachypnea Acidosis	1	Vomiting Tachypnea Acidosis	N.D.[a] (monocyte)
2 (10, F)	1 d	Ketosis Acidosis Hypoglycemia	10	Convulsion	N.D. (lymphocyte, liver)
3 (7, M)	1 y	Hypoglycemia Convulsion	1	Hypoglycemia Convulsion	N.D. (monocyte)
4 (20, F)	1 y	Convulsion Hypoglycemia Hypoglycemia	16	Fever Drowsiness	N.D. (monocyte)

[a] N.D., not detected.

was based on the lack of FDPase activity in lymphocytes, monocytes or liver tissue.

2.6.1. Case 1

A 2-year-old boy: on the 2nd postnatal day, hyperventilation, vomiting, lactic acidemia, ketonuria and hypoglycemia were noted. After the neonatal period, hypoglycemic episodes occurred occasionally and the final diagnosis was made when he was 1 year old. Now at age 2 years, growth and development are normal.

2.6.2. Case 2

A 10-year-old girl: her parents were consanguineous. On the 1st postnatal day, hypoglycemia and metabolic acidosis became apparent. She had repeated episodes of severe abdominal pain, vomiting, metabolic acidosis and hypoglycemia. At age 10 years, convulsion and unconsciousness suddenly occurred early in the morning. She was diagnosed as FDPase deficiency after this attack.

2.6.3. Case 3

A 7-year-old boy: his parents were consanguineous and his sister also had FDPase deficiency. The first attack of hypoglycemia was noted at age 1, and since then similar episodes have occurred occasionally. At age 1 year, diagnosis was established based on the lack of FDPase activity. Now at age 7, he shows severe retardation in growth, development and intelligence.

2.6.4. Case 4

A 20-year-old female: at age 1 year, the first attack of hypoglycemia was noted and similar attacks have occurred occasionally. She did not experience any attacks between the ages of 6 and 15 years. At age 16, an episode of hypoglycemia and drowsiness occurred and the enzymatic and genetic diagnoses were made.

We also analyzed specimens from five patients with GK deficiency for whom the diagnosis was based on clinical manifestations, organic acid analysis by GC–MS and enzyme assay, and ten patients who were undergoing glycerol infusion. Urine samples collected from 30 healthy children served as normal controls.

2.7. Statistics

The *F* test was used to determine statistically significant differences.

3. Results

3.1. Comparison of recovery of compounds between urease/direct method and column method

Fig. 1 shows the recovery rate of compounds, using the urease/direct and column methods and an 8 time inter-assay of the standard solution. Quantification is expressed as the PRA (%) of Q-ion of each compound to that of PDA. The recovery rates of glycerol and 3HB were much higher with the urease/direct method than with the column method ($P<0.001$). No other significant differences were detected among other compounds.

3.2. Comparison of C.V. value between urease/direct method and column method

As shown in Table 3, the C.V. values (%) of all four compounds tended to be smaller in the case of the urease/direct method. In particular, the C.V. value

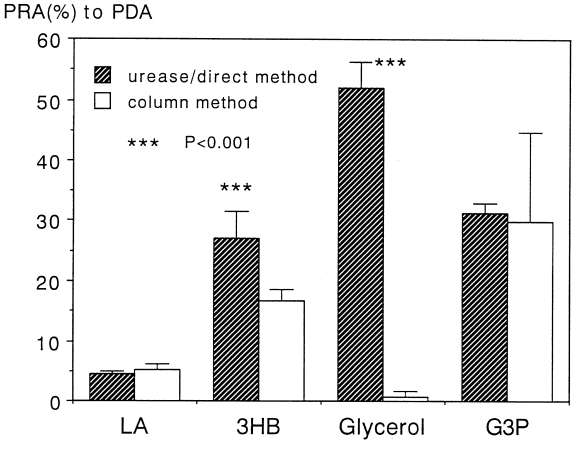

Fig. 1. Recovery rate of compounds, using urease/direct and column methods. Quantification expressed as PRA (%) of Q-ion to that of PDA. Abbreviations: LA, lactic acid; 3HB, 3-hydroxybutyric acid; G3P, glycerol-3-phosphate.

Table 3
C.V. values obtained by different methods[a]

	Urease/direct method		Column method	
	Mean±SD	C.V. (%)	Mean±SD	C.V. (%)
Lactic acid	3.9±0.7	5.6	5.6±0.9	16.7
3-Hyroxybutyric acid	19.7±1.5	7.7	17.4±1.6	9.5
Glycerol	43.5±1.4	3.1	0.8±0.1	11.4
Glycerol-3-phosphate	25.9±1.1	4.3	31.3±6.7	21.3

[a] Quantification was done for 8 time inter-assays, and expressed as PRA (%) of each Q-ion to that of TA (*m*/*z* 299).

of G3P was significantly smaller in the case of the urease/direct method.

3.3. Sensitivity and reproducibility of quantification

As shown in Table 4, the lower limits of detection, using the urease/direct method in the scan mode were ≈10 nmol/ml for LA, 3HB and glycerol, and 20 nmol/ml for G3P. When analyzed in the SIM mode, the lower limits were at least 1 nmol/ml for LA, 3HB and glycerol, and 2 nmol/ml for G3P, using urease/direct method. On the other hand, using the column method and GC–MS-SIM, the lower limit of detection was ≈5 nmol/ml for LA and 3HB, and 10 nmol/ml for glycerol and G3P. As shown in Fig. 2, the linearity of quantification, using the urease/direct method with GC–MS-SIM, was excellent above the lower limit of detection to 500 nmol/ml for each compound. Using the column method, linearity was evident from the lower limit to around 100 nmol/ml for each compound. Thus, the sensitivity, reproductivity and dynamic range of quanti-

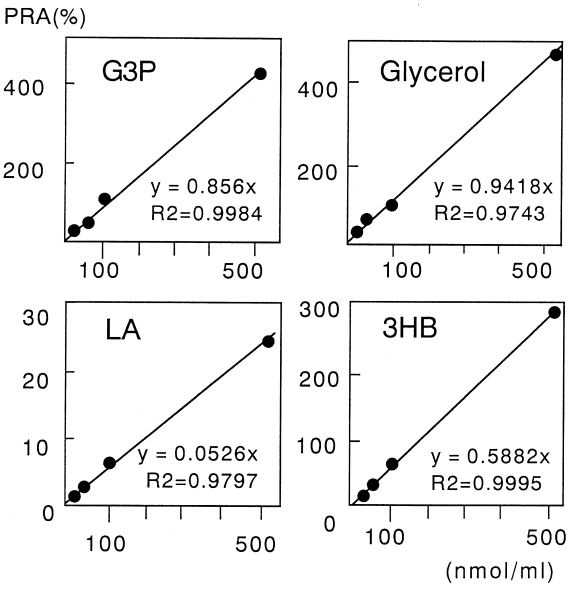

Fig. 2. Linearity of quantification of compounds tested, using urease/direct method and GC–MS-SIM. Quantification expressed as PRA (%) to TA as internal standard. Abbreviations: LA, lactic acid; 3HB, 3-hydroxybutyric acid; G3P, glycerol-3-phosphate.

Table 4
Comparison of lower limits of detection (nmol/ml)[a]

	Urease/direct method		Column method
	SCAN mode	SIM mode	SIM mode
Lactic acid	10	1	5
3-Hydroxybutyric acid	10	1	10
Glycerol	10	1	10
Glycerol-3-phosphate	20	2	10

[a] Numbers represent rough values from authentic standards.

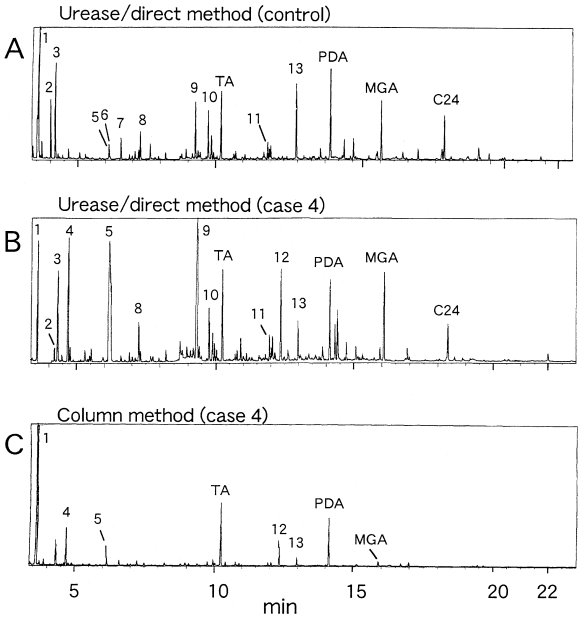

Fig. 3. Total ion current (TIC) chromatogram by GC–MS analysis in the SCAN mode of normal control and a FDPase deficiency patient. (A) Normal control using urease/direct method and GC–MS-SIM (B) FDPase deficiency patient (case 4) using urease/direct method and GC–MS-SIM (C) same sample as in (B) using column method and GC–MS-SIM. Peaks: 1=lactic acid; 2=alanine; 3=glycine; 4=3-hydroxybutyric acid; 5=glycerol; 6=phosphoric acid; 7=succinic acid; 8=serine; 9=erythritol; 10=cysteine; 11=arabitol; 12=glycerol-3-phosphate; 13=citric acid.

fication were better using the urease/direct method with GC–MS-SIM.

3.4. Analysis of patients with FDPase deficiency, GK deficiency, and glycerol infusion patients

We analyzed twelve specimens from four patients with FDPase deficiency, as well as five specimens from five patients with GK deficiency, ten patients undergoing glycerol infusion and thirty controls using the urease/direct method and GC–MS-SIM. Fig. 3 shows total ion current (TIC) chromatograms analyzed in the scan mode of a typical normal control (Fig. 3A) and case 4 in the acute stage (Fig. 3B and C). TIC chromatograms of Fig. 3A and B were after the urease/direct method, and those of Fig. 3C were after the column method. As shown in Fig. 3B and C, the peaks of 3HB (peak 4), glycerol (peak 5) and G3P (peak 12) were higher than those of the normal control shown in Fig. 3A. The glycerol peak (peak 5) was higher in Fig. 3B than that in Fig. 3C.

Glycerol and G3P were detected in all 30 controls in the SIM mode (Table 5). On the other hand, in the scan mode, glycerol was detectable in only 3 of 30 controls, and G3P was detectable in 11 of 30 controls (data not shown). In our method with the SIM mode, urinary levels of glycerol and G3P in normal controls were calculated to be 38.1 ± 13.4 mmol/mol creatinine (range, 16.6–79.2) and 49.4 ± 28.1 (range, 9.3–101.4), respectively.

In three FDPase deficient patients (cases 1, 2 and 4), urinary excretion of LA, 3HB, glycerol and G3P was markedly increased during episodes of hypoglycemia. Excretion of LA, glycerol and G3P was also high, even in the stable state of two subjects (cases 1 and 2), in whom age at onset was in the neonatal period. The concentration of these metabolites in the case 3 subject was within the normal range. However, only one sample taken during a stable condition was available in this case.

The concentration of glycerol was markedly elevated in all five patients with GK deficiency and all ten patients undergoing glycerol infusion. In the glycerol infused patients, excretion of G3P was also increased, whereas in GK deficiency patients,G3P was within the normal range.

The glycerol/G3P molar ratio in FDPase deficiency patients ranged from 0.47 to 1.76, regardless of an acute or stable condition, which is similar to that in the normal controls, 1.0 ± 0.1 (range, 0.9–1.1). In contrast, the ratio of both GK deficiency and glycerol infusion patients was significantly elevated, 145.9 ± 5.5 (range, 72.4–196.7) and 14.1 ± 15.1 (range, 3.3–37.5), respectively. Hence, a clear difference in the glycerol/G3P ratio was observed among the three groups.

4. Discussion

FDPase, a key enzyme in gluconeogenesis catalyzes fructose-1,6-diphosphate to fructose-6-phosphate, and regulates the endogenous formation of glucose from lactate, glycerol or gluconeogenic amino acids. Therefore, during crisis in FDPase deficiency patients, profound hypoglycemia, lactic

Table 5
Urinary organic acids and glycerol-3-phosphate

	Clinical findings at sampling	LA	3HB	glycerol	G3P	glycerol/G3P
FDPase deficiency						
Case 1 (2 y, M)						
(1) Acute	Convulsion, hypoglycemia	361.7	763.3	1110.0	1214.0	0.91
(2) Recovery	2 days after hypoglycemia	1090.5	57.2	116.3	190.7	0.61
(3) Stable	Complicated asthma attack	352.4	38.5	61.4	150.9	0.47
(4) Stable	No symptoms	1028.3	8.0	102.3	177.3	0.57
Case 2 (10 y, F)						
(5) Acute	Convulsion, hypoglycemia	3387.1	80.6	3193.2[a]	1374.0	2.32[a]
(6) Acute	Fructose loading	1527.6	0	445.3	449.2	0.98
(7) Stable	No symptoms	198.8	25.8	98.6	102.5	0.96
Case 3 (7 y, M)						
(8) Stable	No symptoms	15.8	32.0	4.8	4.6	1.03
Case 4 (20 y, F)						
(9) Acute	5 h after hypoglycemia	2049.0	246.6	559.1	477.3	1.17
(10) Recovery	16 h after hypoglycemia	1066.3	132.2	149.3	18.3	0.81
(11) Recovery	21 h after hypoglycemia	810.7	78.0	40.2	22.8	1.76
(12) Recovery	28 h after hypoglycemia	168.0	942.8	22.0	16.5	1.33
GK deficiency (n=5)		558.3±474.2 (69.5–1290.1)	3.2±7.0 (0–16.0)	9101.1±3606.2 (460.9–12260.4)	61.3±10.0 (48.1–75.1)	145.9±45.5 (72.4–196.7)
Glycerol infusion (n=5)		654.4±657.5 (70.2–1641.5)	23.2±34.6 (0–103.6)	324.4±2116.1 (514.3–7238.3)	421.0±410.3 (96.7–1140.1)	14.1±15.1 (3.3–37.5)
Control (n=5)		22.4±15.3 (0.8–70.2)	5.8±15.6 (0–58.8)	38.1±13.4 (16.6–79.2)	49.4±28.1 (9.3–101.4)	1.0±0.1 (0.9–1.1)

[a] Glycerol infusion as a treatment; units: mmol/mol creatinine.

acidosis or ketoacidosis often occur, and unconsciousness or sudden death may follow. Affected infants often become symptomatic during the neonatal period when they are dependent on gluconeogenesis. Although FDPase deficiency has been considered a rare disease, the numbers may be considerably higher if some who died suddenly of unknown causes or those misdiagnosed as Reye's syndrome, epilepsy or ketotic hypoglycemia are included.

Diagnosis of this disorder has generally been made by measurement of enzyme activity in the liver [9], but this technique is invasive and time-consuming. Other studies have suggested that diagnosis can be made using peripheral leukocytes [10], cultured lymphocytes [11] or monocytes [12]. However, Shin reported that the peripheral leukocyte FDPase activity was normal in three of eleven patients with hepatic FDPase deficiency [13].

Although the fructose loading test can be diagnostic, it is not always safe and should not be carried out during acute stages. Patients with FDPase deficiency often present with symptoms of encephalopathy or encephalitis, and glycerol may be often used as treatment or as a prophylaxis of brain edema. However, glycerol administration is contraindicated for FDPase deficiency patients.

Increased excretion of sugar phosphates such as G3P, as well as glycerol has been reportedly observed during an episode in such patients [5–7]. Detection of these metabolites could be an indicator in diagnosis. Using the urease/direct method and GC–MS-SIM analysis, we obtained more sensitive and reproducible results compared to the column method. The reported method is simpler and less time intensive than the column method which requires delicate pH adjustments.

Since both G3P and glycerol were detected in all

the controls using the method described herein, we studied excretion during the acute and stable conditions of four patients with FDPase deficiency, while referring to findings in patients with GK deficiency and glycerol infusion that are associated with hyperglyceroluria. Excretion of G3P and glycerol was elevated in all samples during crisis, and also in many samples taken during recovery or stable phases. Glycerol excretion was also high in subjects with GK deficiency and from glycerol infused patients. Moreover, the glycerol/G3P molar ratio in FDPase deficient patients was within the normal range whereas that in GK deficiency and glycerol infused patients was high, indicating that the glycerol/G3P molar ratio may be a good indicator for a differential diagnosis.

The gene encoding human FDPase has been cloned, sequenced and mapped to chromosome 9q22.2–q22.3 [14], and genetic abnormalities within the FDPase gene have been identified [15,16]. Kikawa et al. identified relatively common mutations, 960/961insG and G164S, in Japanese patients [15]. While screening of FDPase deficiency patients and detection of heterozygotes may be feasible, not all patients can be included in the genetic screening. Therefore, this simple and rapid method is of important clinical significance for early diagnosis of this disorder and for prevention of episodes, particularly if it is combined with screening for gene mutations.

Acknowledgements

We are grateful to Dr. M. Adachi (Kanagawa Prefectural Children's Medical Center, Yokohama, Japan), Y. Hasegawa (Kiyose Children's Hospital, Tokyo, Japan), and H. Sasaki (Wakayama Prefectural Medical University, Wakayama, Japan) for providing urine samples and clinical data. This study was supported in part by a Grant-in-Aid for Research from the Ministry of Education, Science and Culture of Japan and by a Research Grant for Inherited Metabolic Disease from the Ministry of Health and Welfare of Japan and by a grant of the Shimane Institute of Health Science.

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