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Pitfall in the Diagnosis of Fructose-1,6-Bisphosphatase Deficiency: Difficulty in Detecting Glycerol-3-Phosphate with Solvent Extraction in Urinary GC/MS Analysis

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Fructose-1,6-bisphosphatase (FBPase), an enzyme involved in gluconeogenesis, catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate. FBPase deficiency is an autosomal recessive inherited disorder, characterized by episodic attacks of hypoglycemia, ketosis, and lactic acidosis during fasting. In general, urinary organic acid analysis using gas chromatography-mass spectrometry (GC/MS) is very useful for the diagnosis of FBPase deficiency, because the appearance of glycerol or glycerol-3-phosphate in the urine is characteristic of this disease. Here, we report a case of FBPase deficiency in a girl with a history of several severe lactic acidosis events, both as a neonate and after the age of 12 months. The patient was identified as a compound heterozygote with two mutations in the FBPase 1 gene: c.841G>A (p.Glu281Lys) and c.960_961insG (p.Ser321fs). The c.841G>A is a newly identified pathogenic mutation. An abnormal level of glycerol-3-phosphate was not detected in the conventional urinary organic acid analysis using GC/MS after solvent extraction. This method, which is a widely used diagnostic standard, could not detect increased levels of glycerol or glycerol-3-phosphate in the patient's urine, which was sampled during the episode. However, glycerol and glycerol-3-phosphate were detected in the same sample, when it was analyzed using GC/MS with the urease pretreatment non-extraction method. Patients with FBPase deficiency have good glycemic control after correct treatment. Therefore, accurate and early diagnosis is essential for a good prognosis. Accordingly, when a patient presents with hypoglycemia and lactic acidosis, it is important to select the appropriate method of urinalysis for organic acids by GC/MS.

Keywords: fructose-1,6-bisphosphatase deficiency; GC/MS analysis; gluconeogenesis; hypoglycemia; lactic acidosis
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Introduction

Fructose-1,6-bisphosphatase (FBPase; E.C.3.1.3.11) is a key enzyme in gluconeogenesis that catalyzes the hydrolysis of fructose-1,6-bisphosphate into fructose-6-phosphate and inorganic phosphate. FBPase deficiency (OMIM #229700) is an autosomal recessive inherited disorder with the frequency of 1-9/100,000 (Douillard et al. 2012), characterized by episodic spells of hypoglycemia, ketosis, and lactic acidosis during fasting (Baker and Winegrad 1970). In approximately one half of cases, the disease presents during the neonatal period with tachypnea, dyspnea, apneic episodes, tachycardia, irritability, coma, muscular hypotonia, hepatomegaly, hypoglycemia, severe metabolic acidosis, and hyperlactacidemia. Fasting and febrile episodes are known to trigger these symptoms during infancy and childhood. Gas chromatography-mass spectrometry (GC/MS) analysis of urinary organic acids during a spell can be especially helpful when diagnosing FBPase deficiency, because the appearance of glycerol or glycerol-3-phosphate in the urine is characteristic of this disease (Dremsek et al. 1985; Burlina et al. 1990; Nakai et al. 1993). For this method, organic solvent extraction is usually employed for the pretreatment of samples, because target substances such as organic acids are easily collected and contamination of GC column can be prevented (Kimura et al. 1999). On the

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other hand, urease pretreatment non-extraction (urease/direct) method is mainly used for GC/MS metabolome analysis, which enables simultaneous analysis for not only organic acids, but also amino acids, sugar, purine and pyrimidines (Matsumoto and Kuhara 1996; Iga et al. 2000). But this method is not common for ordinary organic acid analysis because peak detection is cumbersome and column contamination is concerned.

We encountered a case of FBPase deficiency in a girl who presented with severe lactic acidosis as a neonate. In this case, a conventional analysis of urinary organic acids by GC/MS after solvent extraction could not detect abnormal excretion of glycerol or glycerol-3-phosphate.

Case Presentation

The patient was the first female child born to healthy Japanese parents after a normal pregnancy and delivery. She showed tachypnea and retraction 24 hours after birth, and was sent to a neonatal intensive care unit. On admission to the hospital, she showed severe hypoglycemia with lactic acidosis (blood glucose; 0.55 mmol/L = 10 mg/dl, pH; 7.16, base excess (BE); −21.9 mmol/L, HCO_3^-; 3.5 mmol/L, lactate; 23.3 mmol/L). Other data included pyruvate 0.58 mmol/L, lactate/pyruvate ratio 40, total ketone bodies 1,386 μmol/L, acetoacetate 217 μmol/L, 3-hydroxybutyrate 1,169 μmol/L, white blood cell count 17,400 /μL, C-reactive protein (CRP) 4.37 mg/dL, and ammonia 101 μg/dL. After admission, she was injected with glucose and sodium hydrogen carbonate. Although the hypoglycemia and pH findings improved after the injection, BE did not improve; hence, continuous hemodiafiltration (CHDF) was performed the next day, which improved the patient's acidosis.

An amino-acid analysis of the blood, taken on the first day after her birth, showed no abnormal results. A urinary organic acid analysis by GC/MS was outsourced to a laboratory company with the sample taken on the third day after birth, which indicated high lactate levels and ketosis. However, an obvious excretion of glycerol-3-phospate was not detected. Analysis of the serum acylcarnitine profile by tandem mass spectrometry (MS/MS) indicated no specific result.

We considered the lactic acidosis to be the result of an inborn metabolic disease; therefore, vitamin B_1, vitamin B_{12}, biotin, and L-carnitine were administered. The patient's condition improved, and feeding of milk did not change her condition. On the twenty-second day after her birth, she was sent to our hospital for continuous treatment. Based on the first urinary GC/MS analysis, we believed that she did not have organic acidurias and stopped the administration of vitamin B_1 and biotin. Afterwards, her condition did not change. On the fifty-ninth day after her birth, she left our hospital.

After leaving the hospital, the patient did not experience lactic acidosis for a year. However, she had experienced episodes of lactic acidosis and hypoglycemia after her first birthday, early in the morning, once every 3 months. In these attacks, levels of liver enzymes, pyruvate and ketone bodies were elevated. The same as before, urine organic acid analyses by GC/MS were repeated, which indicated high lactate and pyruvate excretion and severe ketosis. Because severe lactic acidosis with high lactate/pyruvate ratio and hypoglycemia was observed, we considered a mitochondrial respiratory chain disorder as a possible diagnosis, but the levels of mitochondrial enzymes in biopsied samples of the muscle and liver were normal.

Glycerol loading test

Although excretions of glycerol-3-phosphate in urinary GC/MS analysis were not obvious, severe hypoglycemia with elevation of lactate level suggested disorder of gluconeogenesis, so a glycerol tolerance test was performed after we received informed consent.

The patient was administered glycerol (1 g/kg) after 10 hours of fasting. Blood samples were assayed for glucose, phosphorus, pH, lactate, and pyruvate levels. The glucose level, phosphorus level, and pH decreased, and the lactate level increased (Fig. 1).

Gene analysis

The results of the tests and the episodes indicated FBPase deficiency, so analysis for enzyme activity was performed. Low FBPase activity in cultured monocytes (Kikawa et al. 1993) confirmed the diagnosis. Informed consent was obtained from her parents. The *FBPase 1* (*FBP1*) mutation analysis revealed that she is a compound heterozygote with 2 mutations: c.841G>A (p.Glu281Lys) and c.960_961insG (p.Ser321fs). The c.841G>A was not reported before, but PolyPhen-2, an online site for prediction of functional effects of human mutation, showed 100% specificity for pathogenic mutation. The c.960_961insG was reported before, which is the commonest mutation in Japanese patients (Kikawa et al. 1997).

Reevaluation of urinary GC/MS analysis

To evaluate the results of the urinary GC/MS analysis that was performed previously, we analyzed the stored urine collected during the last episode by GC/MS using the urease/direct preparation method, instead of solvent extraction, and found abnormal amounts of glycerol and glycerol-3-phosphate without a remarkable increase of 2-oxoglutaric acid or α-ketoglutaric acid (Fig. 2). The detected values of lactate, glycerol, and glycerol-3-phosphate were 250-fold, 970-fold and 290-fold greater than the upper limit of normal ranges, respectively (reference values of lactate, glycerol, and glycerol-3-phosphate are < 100 μg/mgCr, < 20 μg/mgCr, and < 2 μg/mgCr, respectively). To compare the urease/direct method and solvent extraction for GC/MS, we reexamined the same sample with the solvent extraction method (Fig. 2B). The resulting chromatogram showed a glycerol peak, but did not show abnormal quantities of glycerol-3-phosphate.

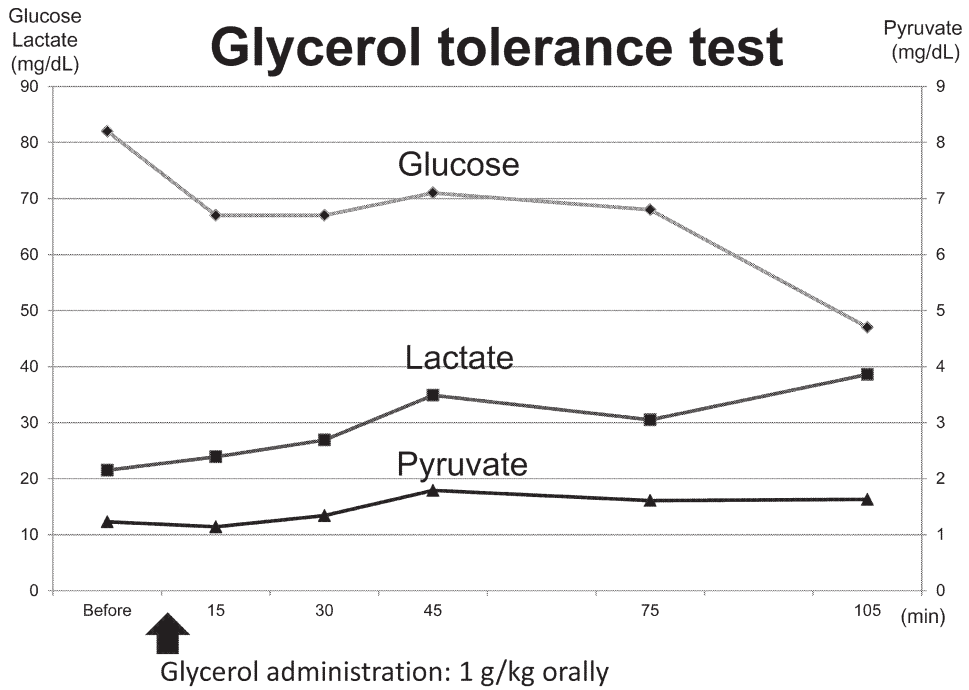

Fig. 1. Glycerol tolerance test.
One g/kg glycerol was administered orally and blood samples were collected at before, 15 min, 30 min, 45 min, 75 min and 105 min after administration and glucose, lactate, and pyruvate levels were analysed. Decrease of glucose level with lactate elevation was observed.

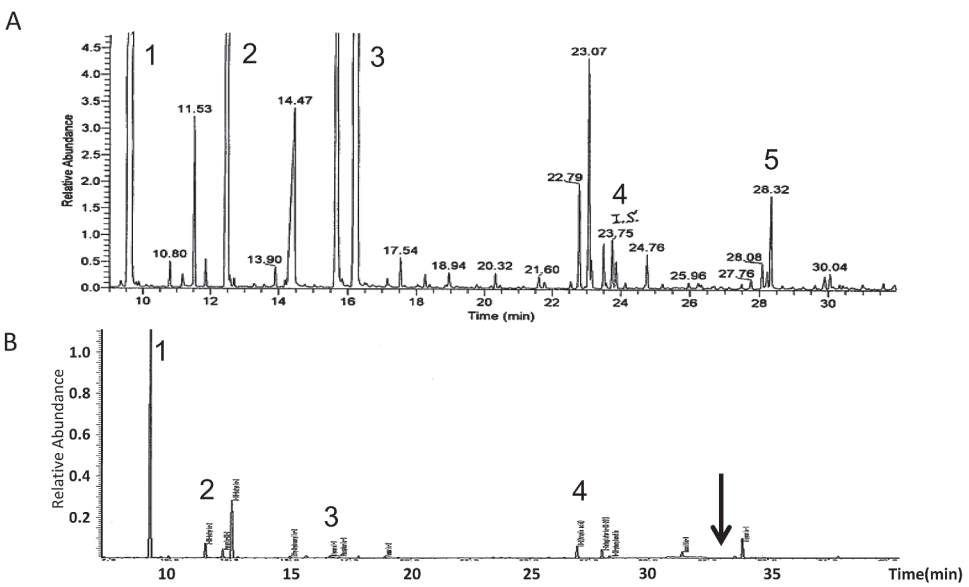

Fig. 2. Total ion chromatograms of GC/MS urine analysis.
A; urease/direct method. B; solvent extraction method.
The chromatogram in panel B showed low glycerol levels without glycerol-3-phosphate peak. The arrow (↓) indicates the location at which the glycerol-3-phosphate peak should be detected.
Peak identifiers: lactate (1), 3-hydroxy butyrate (2), glycerol (3), internal standard (4), and glycerol 3-phosphate (5).

After diagnosis, we stopped the administration of vitamins and carnitine, and initiated a supplement of special formula for glycogen storage disease before sleep to prevent hypoglycemia early in the morning. The frequency of lactic acidosis episodes decreased, and the patient currently displays good development.

Discussion

FBPase deficiency is characterized by episodic spells of hypoglycemia, ketosis, and lactic acidosis during fasting or infection. Patients have good prognosis once FBPase deficiency has been diagnosed, and adequate management is introduced. The growth and development of patients are normal, and tolerance to fasting improves with age; subsequently, the disorder shows almost no problem later in life even in pregnant women under prompt medical intervention (Sugita et al. 2013). Hence, correct diagnosis of early episodes is very important.

Patients with FBPase deficiency present with hypoglycemia and high lactate levels, but these data are not specific and it is difficult to distinguish FBPase deficiency from other inborn errors of metabolism. To diagnose this disease, detection of characteristic metabolites, that is glycerol and glycerol-3-phosphate in the urinary sample collected during a spell is quite helpful. Particularly, glycerol-3-phosphate is more specific because glycerol is easily detected as a result of medication, which is a main component of enemas and salves that are widely used for children.

GC/MS analysis of organic acids is usually performed after extraction of the organic solvent from the samples, because the target compounds are efficiently extracted and GC/MS columns are protected from contamination. Glycerol and glycerol-3-phosphate are usually detectable with this method, but in our case, the urine samples taken during the episode did not indicate obvious excretion of glycerol-3-phosphate. However, glycerol and glycerol-3-phosphate were detected in the same urine by GC/MS with the urease/direct preparation. In this method, many substances, which are hardly soluble in organic solvent, are detected because removal of urea by urease treatment enables direct injection of urine sample to GC/MS system. Using the solvent extraction method, glycerol and lactate results were 122 and 237 times their normal maximum values, respectively. However, using the urease/direct preparation, glycerol and lactate results were 974 and 252 times their normal maximum values, respectively. We suspected that high lactate levels might prevent the extraction of glycerol or glycerol-3-phosphate from the urine.

Patients with FBPase deficiency may have a high lactate/pyruvate ratio. This may be a consequence of secondary impairment of the conversion of 1,3-bisphosphoglycerate into glyceraldehyde-3-phosphate, resulting in accumulation of NADH, which is the other substrate of glyceraldehyde-3-phosphate dehydrogenase (van den Berghe 1996). On the other hand, lactic acidosis with an elevated lactate/pyruvate ratio is a hallmark of mitochondrial respiratory chain disorders, which are more common than FBPase deficiency. Mochel et al. (2005) reported that hypoglycemic patients with respiratory chain defects and mitochondrial respiratory chain disorders experience a variety of symptoms.

It is difficult to diagnose FBPase deficiency when glycerol or glycerol-3-phosphate is not detected in the urine. We were unable to diagnose the condition before a glycerol tolerance test, but administration of glycerol to the patients with FBPase deficiency can be harmful (Hasegawa et al. 2003) and should be avoided, if possible. Therefore, the detection of glycerol or glycerol-3-phosphate in the urine is very important for the safety of patients as well as for the diagnosis of FBPase deficiency. Patients with FBPase deficiency have good glycemic control after being provided with the correct treatment and show normal development. Hence, the diagnosis of FBPase deficiency is very important for their future lives as well. To evaluate hypoglycemic patients associated with lactic acidosis, careful attention should be paid to the method of sample preparation for analysis of organic acids in the urine by GC/MS and if high lactate excretion was observed, re-examination with urease/direct method should be considered.

Conflict of Interest

The authors declare no conflict of interest.

References

Baker, L. & Winegrad, A.I. (1970) Fasting hypoglycaemia and metabolic acidosis associated with deficiency of hepatic fructose-1,6-diphosphatase activity. *Lancet*, **2**, 13-16.

Burlina, A.B., Poletto, M., Shin, Y.S. & Zacchello, F. (1990) Clinical and biochemical observations on three cases of fructose-1,6-diphosphatase deficiency. *J. Inherit. Metab. Dis.*, **13**, 263-266.

Douillard, C., Mention, K., Dobbelaere, D., Wemeau, J.L., Saudubray, J.M. & Vantyghem, M.C. (2012) Hypoglycaemia related to inherited metabolic diseases in adults. *Orphanet J. Rare Dis.*, **7**, 26.

Dremsek, P.A., Sacher, M., Stögmann, W., Gitzelmann, R. & Bachmann, C. (1985) Fructose-1,6-diphosphatase deficiency: glycerol excretion during fasting test. *Eur. J. Pediatr.*, **144**, 203-204.

Hasegawa, Y., Kikawa, Y., Miyamaoto, J., Sugimoto, S., Adachi, M., Ohura, T. & Mayumi, M. (2003) Intravenous glycerol therapy should not be used in patients with unrecognized fructose-1,6-bisphosphatase deficiency. *Pediatr. Int.*, **45**, 5-9.

Iga, M., Kimura, M., Ohura, T., Kikawa, Y. & Yamaguchi, S. (2000) 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. *J. Chromatogr. B Biomed. Sci. Appl.*, **746**, 75-82.

Kikawa, Y., Inuzuka, M., Jin, B.Y., Kaji, S., Koga, J., Yamamoto, Y., Fujisawa, K., Hata, I., Nakai, A., Shigematsu, Y., Mizunuma, H., Taketo, A., Mayumi, M. & Sudo, M. (1997) Identification of genetic mutations in Japanese patients with fructose-1,6-bisphosphatase deficiency. *Am. J. Hum. Genet.*, **61**, 852-861.

Kikawa, Y., Takano, T., Nakai, A., Shigematsu, Y. & Sudo, M. (1993) Detection of heterozygotes for fructose-1,6-diphosphatase deficiency by measuring fructose-1,6-diphosphatase activity in monocytes cultured with calcitriol. *Clin. Chim. Acta*, **215**, 81-88.

Kimura, M., Yamamoto, T. & Yamaguchi, S. (1999) Automated metabolic profiling and interpretation of GC/MS data for organic acidemia screening: a personal computer-based system. *Tohoku J. Exp. Med.*, **188**, 317-334.

Matsumoto, I. & Kuhara, T. (1996) A new chemical diagnostic

method for inborn errors of metabolism by mass spectrometry: rapid, practical, and simultaneous urinary metabolites analysis. *Mass Spectrom. Rev.*, **15**, 43-57.

Mochel, F., Slama, A., Touati, G., Desguerre, I., Giurgea, I., Rabier, D., Brivet, M., Rustin, P., Saudubray, J.M. & DeLonlay, P. (2005) Respiratory chain defects may present only with hypoglycemia. *J. Clin. Endocrinol. Metab.*, **90**, 3780-3785.

Nakai, A., Shigematsu, Y., Liu, Y.Y., Kikawa, Y. & Sudo, M. (1993) Urinary sugar phosphates and related organic acids in fructose-1,6-diphosphatase deficiency. *J. Inherit. Metab. Dis.*, **16**, 408-414.

Sugita, G., Tsuyoshi, H., Nishijima, K. & Yoshida, Y. (2014) Fructose-1,6-bisphosphatase deficiency: a case of a successful pregnancy by closely monitoring metabolic control. *JIMD Rep.*, **14**, 115-118.

van den Berghe, G. (1996) Disorders of gluconeogenesis. *J. Inherit. Metab. Dis.*, **19**, 470-477.