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Review

Noninvasive human metabolome analysis for differential diagnosis of inborn errors of metabolism☆

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Abstract

Early diagnosis and treatment are critical for patients with inborn errors of metabolism (IEMs). For most IEMs, the clinical presentations are variable and nonspecific, and routine laboratory tests do not indicate the etiology of the disease. A diagnostic procedure using highly sensitive gas chromatography–mass spectrometric urine metabolome analysis is useful for screening and chemical diagnosis of IEM. Metabolite analysis can comprehensively detect enzyme dysfunction caused by a variety of abnormalities. The mutations may be uncommon or unknown. The lack of coenzymes or activators and the presence of post-translational modification defects and subcellular localization abnormalities are also reflected in the metabolome. This noninvasive and feasible urine metabolome analysis, which uses urease-pretreatment, partial adoption of stable isotope dilution, and GC/MS, can be used to detect more than 130 metabolic disorders. It can also detect an acquired abnormal metabolic profile. The metabolic profiles for two cases of non-inherited phenylketonuria are shown. In this review, chemical diagnoses of hyperphenylalaninemia, phenylketonuria, hyperprolinemia, and lactic acidemia, and the differential diagnosis of β-ureidopropionase deficiency and primary hyperammonemias including ornithine transcarbamylase deficiency and carbamoylphosphate synthetase deficiency are described.

Keywords: Metabolome; Chemical diagnosis; GC–MS; Lactic acidemia; Hyperphenylalaninemia; Secondary phenylketonuria; Hyperprolinemia; Hyperammonemias; Ornithine transcarbamylase deficiency

Contents

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1. Introduction

Human urine contains numerous metabolic intermediates at a variety of concentrations and can provide clues that directly lead to diagnoses of enzyme dysfunctions, protein abnormalities, and the related genetic mutations. In short, urine contains the evidence required for the chemical diagnosis of inborn errors of metabolism (IEMs). Many IEMs that are classified as organic acidemias, in which organic acids accumulate in the urine, have been discovered using gas chromatography/mass spectrometry (GC/MS) of organic acids from urine fractionated by solvent extraction, ion exchange with DEAE Sephadex, or silica gel chromatography. The pretreatment of urine with urease has enabled the simultaneous analysis of several categories of compounds [1]. Drawing on our 30 years experience performing chemical diagnoses of IEMs, we developed a practical and drastically simplified urease-pretreatment and GC/MS method [2]. Later, we improved the method by the partial adoption of stable isotope dilution [3,4], and other methodological improvements were made more recently [5,6]. For the evaluation of a metabolite, an abnormality n was defined as n in [mean value of age-matched control above $n \times$ SD]. Mean and SD were obtained after $\log_{10}$ transformation and expressed based on both creatinine and total creatinine (creatinine plus creatine).

This noninvasive, feasible, and cost-effective metabolome analysis of urine, which combines urease-pretreatment, stable isotope dilution, and GC/MS, enables the simultaneous analysis of multiple categories of compounds and offers reliable and quantitative evidence for the screening or chemical diagnosis of more than 130 IEMs [7]. Metabolome analysis is especially applicable to the diagnosis of IEMs in patients early in the course of the disease. Here, we show metabolome profiles in patients with hyperphenylalaninemia, hyperprolinemia types I and II, lactic acidemia, hyperammonemias including ornithine transcarbamylase (OTC) deficiency, propionic acidemias, and methylmalonic acidemias. This noninvasive and feasible metabolic profiling is also useful for detecting acquired metabolic abnormalities. The metabolic profiles of phenylketonuria are shown in two patients without inherited phenylketonuria.

2. Differential diagnoses

2.1. Hyperphenylalaninemia

The major metabolic pathway of phenylalanine is its 4-hydroxylation, which is catalyzed by phenylalanine hydroxylase and yields tyrosine [8]. Hyperphenylalaninemia is caused by either a phenylalanine hydroxylase deficiency or an abnormality in the synthetic pathway of tetrahydrobiopterine (BH_4), a cofactor in the hydroxylase reaction [9]. In the latter case, not only phenylalanine, but also tyrosine and tryptophan metabolism is affected, because BH_4 also functions as the coenzyme of tyrosine hydroxylase to form dihydroxyphenylalanine and tryptophan hydroxylase to form 5-hydroxytryptophan. Thus, these two pathological etiologies are differentiated from the urinary levels of neopterin and biopterin. In phenylalanine hydroxylase deficiency, BH_4-responsive hyperphenylalaninemia or phenylketonuria is seen. In the former the phenylalanine increase is milder than in the latter and phenylalanine hydroxylase protein has a low affinity for the coenzyme or is stabilized by it. In phenylketonuria, a severe form of phenylalanine hydroxylase deficiency, phenylalanine is metabolized via a by-path to give byproducts such as phenylpyruvate, *o*-hydroxyphenylacetate, phenyllactate, phenylacetate, and phenylmandelate. Thus, the severity of the phenylalanine hydroxylase deficiency is reflected in the degree of increased phenylalanine and its byproducts in the urine.

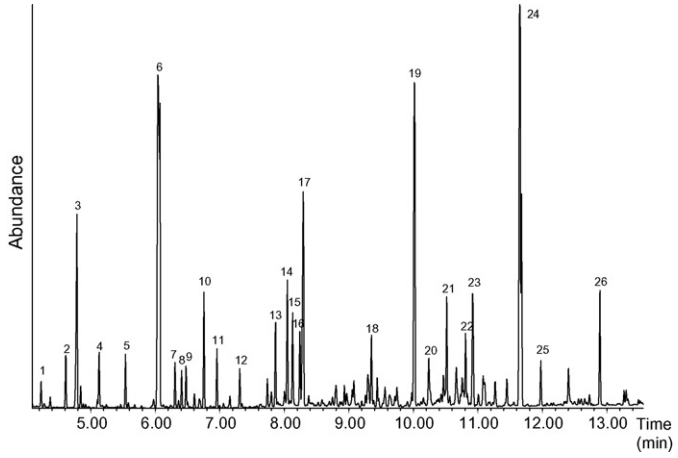

Fig. 1. TIC of trimethylsilyl derivatives of metabolites from the urine of a patient with BH_4-responsive hyperphenylalaninemia. The major component of each major peak is: (1) lactate-2; (2) alanine-2; (3) glycine-2; (4) sulfate-2; (5) β-aminoisobutyrate-2; (6) phosphate-3; (7) proline-2; (8) succinate-2; (9) 2,2-dimethylsuccinate-2 (IS_1); (10) serine-3; (11) threonine-3; (12) 2-deoxytetronate-3; (13) erythritol-4; (14) 4-hydroxyproline-3; (15) erythronate-4(1); (16) threonate-4(2); (17) creatinine-3; (18) xylitol-5; (19) citrate-4; (20) galactose-5(1); (21) galactose-5(2); (22) ascorbate-4; (23) glucose-5(2); (24) myo-inositol-6; (25) *n*-heptadecanoate-1(IS_3); (26) pseudouridine-5.

Urine metabolome analysis was carried out for an infant whose phenylalanine level had been moderately increased as assessed by the current neonatal screening: the Guthrie blood test. The total ion chromatogram (TIC) of trimethylsilyl derivatives of urine metabolome from this case is shown in Fig. 1 and mass chromatograms for the same infant in Fig. 2. The level of phenylalanine in urine taken at 19 days of age and quantified with D_5-phenylalanine as an internal standard was distinctly increased with a mean above 3.7 SD and 3.5 SD for the creatinine-based and the total creatinine-based assays, respectively. Unlike in severe hyperphenylalaninemia, no increase of phenyllactate, *o*-hydroxyphenylacetate, or phenylmandelate was observed. The quantification of phenylpyruvate, keto acid, is not very adequate in the sample preparation. However, an increase in the keto acid is indicated by increased levels of both phenyllactate and the precursor amino acid, phenylalanine. The tyrosine and tryptophan levels were normal, suggesting that an abnormality in the catabolic pathway of these amino acids could be ruled out.

The major catabolic pathway for tyrosine is transamination to form 4-hydroxyphenylpyruvate followed by the further degradation of the organic acid. However, any increase in tyrosine

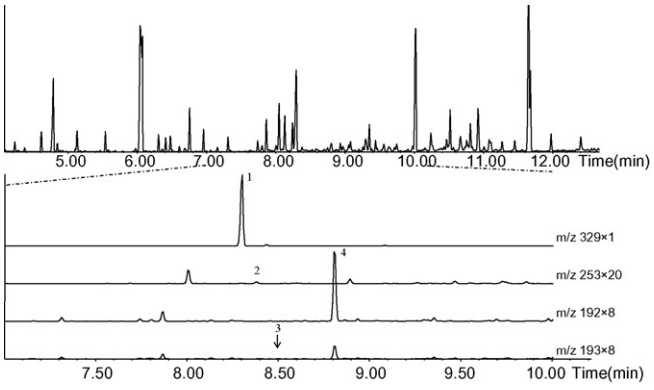

Fig. 2. TIC and mass chromatograms of urinary metabolites from an infant with biopterin-responsive hyperphenylalaninemia. Ions used for quantification are: *m/z* 329 for creatinine (1), *m/z* 253 for *o*-hydroxyphenylacetate (2), *m/z* 192 for phenylalanine (4), and *m/z* 193 for phenyllactate (3).

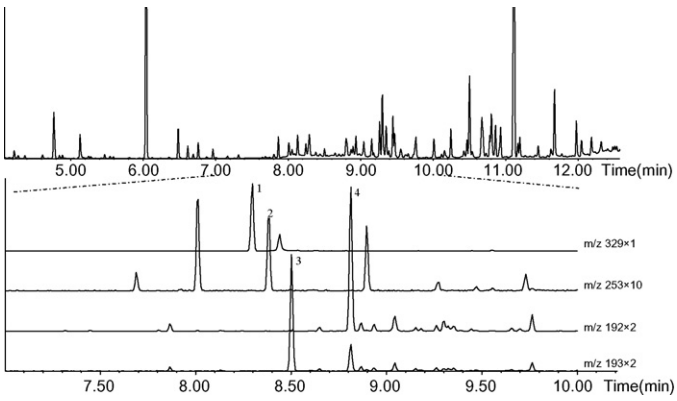

Fig. 3. Metabolic profile of acquired phenylketonuria induced by intravenous hyper-caloric alimentation with Unicalic N containing phenylalanine at the concentration of 2.3 g/L. Ions monitored: *m/z* 329 for creatinine (1), *m/z* 253 for *o*-hydroxyphenylacetate (2), *m/z* 192 for phenylalanine (4), and *m/z* 193 for phenyllactate (3).

catabolic intermediates were not found suggesting the normal tyrosine catabolism. The following investigation of biopterin responsiveness in the patient and the profile analysis of biopterin and neopterin confirmed that this case had biopterin-responsive hyperphenylalaninemia.

2.2. Metabolic profile of secondary phenylketonuria

Metabolome analysis can detect abnormal metabolic profiles derived from inherited mutations or *de novo* mutations. Unlike genome analysis, it can also detect the metabolic abnormality caused by the response of biological systems. Dietary deficiency and nutrient conditions are prime factors that affect the metabolome. Metabolome profiling will be useful to detect acquired abnormalities in the metabolism not only of a few amino acids, but also of a vast number of metabolites of different classes. We found two cases of secondary phenylketonuria: both showed their abnormal profile when they were under intravenous hyper-caloric alimentation with Unicalic N, a fluid containing 2.3 g/L phenylalanine. The first case, an 8-year-old floppy boy with brain atrophy, was under general anesthesia for the control of convulsion. His metabolic profile of phenylketonuria under these special conditions, was not inborn, because he had not phenylketonuria profile examined at the age of 4 years by us and the neonatal Guthrie blood test for him had been negative for hyperphenylalaninemia. As shown in TIC and mass chromatograms (Fig. 3), phenylalanine, *o*-hydroxyphenylacetate, and phenyllactate were markedly increased in his urine, with mean above 5 SD, 8 SD, and 7 SD, respectively. The latter two aromatic acids do not significantly increase in mild hyperphenylalaninemia or biopterin-responsive hyperphenylalaninemia, but do increase in phenylketonuria, in which there is a marked increase of phenylalanine. The analysis of his phenylalanine hydroxylase gene did not show any known mutations. The second case was 11-year-old boy with malignant lymphoma. The metabolic profile of phenylketonuria was detected when he was under intravenous hyper-caloric alimentation and methotrexate treatment, but it disappeared after the removal of intravenous hyper-caloric alimentation and methotrexate that affects phenylalanine hydroxylation.

Intravenous hyper-caloric alimentation alone did not cause a metabolic profile of phenylketonuria, although the phenylalanine concentration in Unicalic N is slightly higher than the recommended levels and the fluid was taken intravenously. It was suggested that the alimentation plus additional factor under diseased or medicated circumstances might be responsible for the PKU profile in these cases.

The metabolic profile of phenylketonuria is caused, not due to an inherited phenylalanine hydroxylase deficiency, but due to the combination of the alimentation and other undetermined factors. Biopterin deficiency was not likely because the urinary biopterin levels were not decreased. It is likely that the amount of phenylalanine exceeded the capacity of the hydroxylase, causing byproducts of phenylalanine to accumulate. Further examination of the background of this metabolic disturbance is under investigation. These case studies are a good example of why urinary metabolome analysis is useful for detecting and understanding metabolic disturbances caused secondarily by medication or special alimentation. These findings also strongly suggest that such a noninvasive urine metabolome analysis is useful for monitoring or evaluating treatments in personalized medicine.

Abnormally elevated levels of phenylalanine and its byproducts may cause brain abnormalities [8]. The degree of the effects may depend on the age of the patients and the duration of the abnormal metabolic response. Fortunately, compounds that are closely related metabolically can be analyzed simultaneously, and no further analyses are required to identify the type of metabolic abnormality. Finally, the accuracy of this diagnostic procedure is supported by the findings from the simultaneous analysis of hundreds of metabolites, which show their levels to be within the known range for healthy control subjects. Noninvasive metabolome profiling may suggest to lower the content of the amino acid leading to the prompt normalization of the metabolic profile.

2.3. Hyperprolinemia

There are two types of hyperprolinemia caused by inherited disorders in proline metabolism. Type I hyperprolinemia

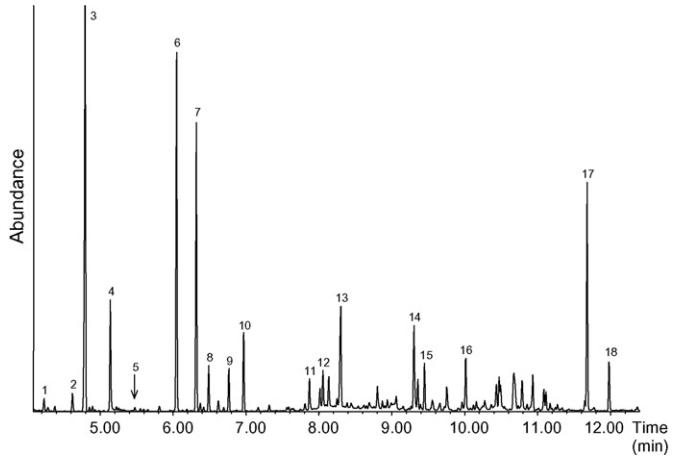

Fig. 4. TIC of trimethylsilyl derivatives of metabolites from the urine of a patient with hyperprolinemia. The major component of each major peaks is: (1) lactate-2; (2) alanine-2; (3) glycine-2; (4) sulfate-2; (5) Δ^1-pyrroline-5-carboxylate; (6) phosphate-3; (7) proline-2; (8) 2, 2-dimethylsuccinate-2 (IS_1); (9) serine-3; (10) threonine-3; (11) erythritol-4; (12) 4-hydroxyproline-3; (13) creatinine-3; (14) d_4-lysine-3 (IS); (15) 2-hydroxyundecanoate-2 (IS_2); (16) citrate-4, (17) urate-4; (18) heptadecanoate-1 (IS_3).

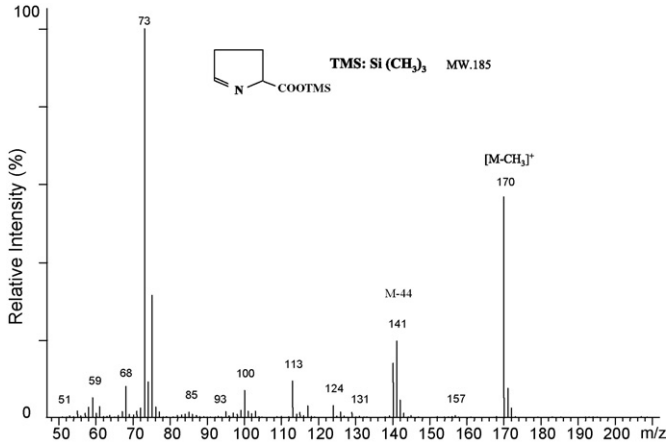

Fig. 5. Mass spectrum of trimethylsilyl derivative of Δ^1-pyrroline-5-carboxylate.

(HPI,MIM 239500) is due to a deficiency of proline oxidase, and type II hyperprolinemia (HPII, MIM 239510) is caused by a deficiency of Δ^1-pyrroline-5-carboxylate dehydrogenase [10]. Both are autosomal-recessive traits. Previous reports demonstrated that HPI is a relatively benign condition, whereas the metabolic abnormality in HPII may cause neurological manifestations such as recurrent and refractory seizures or mental retardation [11]. It is reported that proline at high concentration in plasma or CSF cause brain dysfunction through reduction of creatine kinase or acetylcholinesterase activity [12]. The increase in the proline level is more pronounced in HPII than HPI. The distinguishing biochemical characteristic is the increased urinary excretion of Δ^1-pyrroline-5-carboxylate in patients with HPII [13].

Fig. 4 shows the metabolic profile of a 4-year-old boy with HPII presenting acute encephalopathy associated with influenza virus type A infection [14]. The increase in proline is marked, and hydroxyproline and glycine are significantly increased, but less than proline. Neither glucosuria nor phosphaturia was detected, but Δ^1-pyrroline-5-carboxylate was increased. The mass spectrum of this indicator as a trimethylsilyl derivative is shown in Fig. 5. Δ^1-Pyrroline-5-carboxylate could not be recovered by organic solvent extraction from urine but was recovered well by the method of simplified urease-pretreatment with no fractionation procedure.

The concomitant increase of proline, hydroxyproline, and glycine is known as familial iminoglycinuria, which is autosomal-recessive inborn error of membrane transport but is benign. It involves a membrane carrier in the renal tubule with a preference for L-proline, hydroxy-L-proline, and glycine. The differential diagnosis of familial iminoglycinuria includes hyperprolinemia, Fanconi syndrome, hyperglycinemia, and neonatal hyperiminoglycinuria. Iminoglycinuria occurs as a result of combined saturation-inhibition mechanisms in hyperprolinemia. In Fanconi syndrome, iminoglycinuria occurs as part of a generalized disturbance of transport, including that of amino acids, sugars, and phosphate (In the urinary metabolome analysis, Δ^1-pyrroline-5-carboxylate, amino acids, sugars, and phosphate are also evaluated simultaneously). Fanconi syndrome, as described in fatal infantile mitochondrial myopathy with lactic acidemia and a De Toni–Fanconi–Debré syndrome, in the section on lactic acidemia, shows an amino acid profile different from that of familial iminoglycinuria, hyperprolinemia, hyperglycinemia, and neonatal hyperiminoglycinuria. Accordingly, the urine metabolic profile for the case described above could point toward a diagnosis of HPII.

2.4. Lactic acidemias

Lactic acidemia is defined as a serum lactate concentration above 2 mM. More than 23 etiologies are known to cause lactic acidemia. Primary lactic acidemia includes pyruvate dehydrogenase complex disorders, oxidative phosphorylation (OHPHOS), gluconeogenesis disorders, and membrane transport defects [15]. Eight organic acidemias are the cause of secondary lactic acidemia. These organic acidemias can all be differentially and chemically diagnosed by our metabolome analysis. Among patients with gluconeogenesis disorders, patients with fructose-1,6-diphosphatase deficiency and those with glucose-6-phosphatase deficiency show lactic acidemia during hypoglycemic episodes along with typical urinary metabolic profile: increased lactate, glycerol-3-phosphate and glycerol in fructose-1,6-diphosphatase deficiency and increased lactate and 2-oxoglutalate in glucose-6-phosphatase deficiency.

A typical example that shows the strength of metabolome profiling for chemical diagnosis is primary, chronic lactic acidemia caused by multiple cytochrome deficiencies. This disease, described in fatal infantile mitochondrial myopathy with lactic acidemia and a De Toni–Fanconi–Debré syndrome (MIM 220110), is characterized by floppiness, failure to thrive, and lactic acidosis associated with a biochemical defect in mitochondrial oxidative phosphorylation [16].

Some years ago, we received urine specimens from a 3-month-old floppy infant with severe lactic acidemia, and examined the samples to make chemical diagnosis. The

pathological examination suggested two possibilities: carnitine deficiency or multiple cytochrome deficiency. In those days, the urinary metabolites analyzable by GC/MS were limited to the organic acids that are extractable with an organic solvent under acidic conditions, and amino acids were analyzed separately on an automatic amino acid analyzer. Gross lactic aciduria, phosphaturia, ketonuria, and increased excretion of tricarboxylic acid (TCA) cycle intermediates were observed [17]. In addition, the excretion of fumarate and the ratio of fumarate to succinate were dramatically increased. Organic acidemias and amino acidemias were ruled out. The amino acid excretion was generally increased and the profile was distinctly abnormal and differed from that observed in Hartnup disease, iminoglycinemia, dibasic amino aciduria, or cystinuria. The profile was very similar to that reported by Van Biervliet et al. for De Toni–Fanconi–Debré syndrome: i.e., lactic aciduria and an increased level of intermediary metabolites of the TCA cycle in a floppy baby [16]. On the basis of the profiles of both organic acids and amino acids, the infant was suspected of having a multiple cytochrome deficiency, and was later confirmed to have a cytochrome aa_3/*b* deficiency [18]. Three diagnosed cases from two families including the above case had the characteristic clinical symptoms of severe lactic aciduria, generalized amino aciduria, ketonuria, glucosuria, and phosphaturia [17]. Nowadays, this typical profile could be identified in a single analysis using the noninvasive urine metabolome analysis [19].

2.5. Hyperammonemias and OTC deficiency

There are more than 40 etiologies that result in hyperammonemias: eight are primary hyperammonemias (six urea cycle disorders and two membrane transport disorders), and the rest are secondary hyperammonemias. In the four urea cycle disorders and two membrane transport disorders that lead to primary hyperammonemia, carbamoylphosphate increases because of its reduced or deficient utilization, which leads to increased orotate, orotidine, and uracil. But in the remaining two urea cycle disorders, the synthesis of carbamoylphosphate is greatly reduced or absent, and therefore the levels of orotate, orotidine, and uracil do not increase. Thus, the inability to synthesize carbamoylphosphate can be differentiated from deficient utilization of carbamoylphosphate by the respective low or high urinary levels of orotate and uracil. Diagnostic value of urinary orotate levels for differentiating the hyperammonemia disorders is described [20]. It reduces the need for enzyme determination in tissue biopsies. Urease-pretreatment without fractionation and the adoption of stable isotope dilution to measure orotate and uracil enables the highly sensitive detection of a decrease or increase of these important indicators, and thereby permits sensitive screening for the six primary hyperammonemias [3,4,21], as well as a prompt re-focusing on the two primary hyperammonemias, if the six primary hyperammonemias are ruled out.

In general, a specific enzyme catalyzes metabolite A to B, and the level of A is maintained within the distribution found in healthy individuals. If the enzymatic reaction from A to B is disturbed, substrate A accumulates, resulting in its increase in urine. One exception is ornithine, which cannot be used as a biomarker for a deficiency of OTC (for which ornithine is the substrate), because its level does not increase in the urine or blood in the case of this disorder. The important biomarkers for OTC deficiency include orotate, uracil, pyroglutamate, glutamine, alanine, glycine, proline, β-ureidopropionate, and lysine, of which orotate is the key indicator. Lysinuric protein intolerance can be chemically diagnosed by an increase in the orotate, uracil, and lysine in urine. Although soft ionization mass spectrometric analysis, to quantify citrulline, argininosuccinate, arginine, and homocitrulline, is critical to differentiate argininosuccinate synthetase deficiency, argininosuccinate lyase deficiency, arginase deficiency, and HHH (hyperammonemia, hyperornithinemia, and hyperhomocitrullinemia) syndrome, urinary metabolome analysis is the most sensitive method to screen for OTC deficiency. OTC deficiency is an X-linked disorder and in female carriers, DNA analysis and enzyme determination in tissue biopsies are often difficult to estimate the hepatic enzyme activity. The urinary metabolome profile reflects the patients' hepatic capacity to detoxify ammonia, which indicates the necessity of treatment of the hyperammonemias or the suitability of the mother having an affected female child as a donor in living-related liver transplantation.

It has been generally thought that urinary organic acid profiling by GC/MS is not useful for the diagnosis of the first two urea cycle disorders. The recovery of orotate is low in the organic acid solvent extraction, but it is very high in urease-pretreatment. In the latter GC/MS quantification may lead to an overestimation of urinary orotate, because of the high recovery of orotidine and of the thermal decomposition of orotidine into orotate and sugar. Nevertheless, the highly sensitive orotate evaluation based on the comparison with age-matched control, $\log_{10}$ transformation and out put of abnormality *n* is very helpful for diagnosing the remaining two primary hyperammonemias where the synthesis of carbamoylphosphate is blocked; the method readily detects the lack of increase in the pyrimidine derivatives orotate, orotidine, and uracil that indicates the low carbamoylphosphate level. The information that shows orotate and/or uracil do not increase regardless of an increase in 5-oxoproline, alanine, glutamine, proline, and lysine; information that is simultaneously obtainable from the urease-pretreatment procedure, is very important; it significantly reduces the time required to diagnose these disorders and can lead to an early diagnosis for patients who might otherwise be missed. Thus, urinary metabolome analysis will improve the rate of identifying patients who have, not only the six primary hyperammonemias, but also the remaining two primary hyperammonemias.

We recently evaluated two patients with severe hyperammonemia, at normal or reduced orotate levels, and increased levels of amino acids described above, whom we suspected of having carbamoylphosphate synthetase deficiency (Table 1). Shortly afterwards, a mutation in the carbamoylphosphate synthetase gene was confirmed for both cases. Despite such success, the minor possibility of a deficiency of *N*-acetylglutamate synthetase, which activates carbamoylphosphate synthetase, should always be considered for these profiles, until our current measurement of *N*-acetylglutamate is shown to be efficient for

Table 1
Abnormalities *n* in [mean above *n**SD] in pyrimidine metabolites in urine of patients with metabolic disorders

Compound	Structure	*m/z*	Case 1 βUPD		Case 2 OTCD		Case 3 ASSD		Case 4 ASLD		Case 5 ArginaseD		Case 6 CPSD		Case 7 CPSD	
			GC/MS	Enz	GC/MS	Enz	GC/MS	Enz	GC/MS	Enz	GC/MS	Enz	GC/MS	Enz	GC/MS	Enz
Uracil	M^+	*m/z* 256	1.7	1.4	5.0	4.2	6.0	5.5	5.3	5.7	4.1	6.4	−2.0	−1.8	2.1	3.2
	$[M–H]^+$	*m/z* 255	1.7	1.5	4.9	4.2	5.7	5.3	5.2	5.6	4.1	6.2	−1.3	−1.1	2.2	3.2
	$[M–CH_3]^+$	*m/z* 241	1.9	1.6	5.0	4.3	5.9	5.5	5.2	5.6	4.1	6.3	−1.1	−0.8	2.5	3.8
Orotate	$[M–CH_3]^+$	*m/z* 357	0.2	−0.5	9.2	7.2	10.8	8.8	5.3	6.6	8.8	12.8	−0.2	0.3	0.1	1.4
	$[M–HCOOTMS]^+$	*m/z* 254	0.6	0.0	9.2	7.4	10.6	8.9	5.7	6.8	9.2	12.9	−0.2	0.3	0.1	1.4
5, 6-Dihydrothymine	$[M–H]^+$	*m/z* 271	4.0	2.8												
5, 6-Dihydrouracil	$[M–CH_3]^+$	*m/z* 243	4.5	4.9												
β-Ureidopropionate	$[M–CH_3]^+$	*m/z* 261	7.7	7.7	5.9	5.5	7.2	7.1	3.0	3.6	2.1	4.0				
	$[(CH_3)_2Si=N^+HCON=CH_2]$	*m/z* 129	8.7	8.6	5.2	4.5	6.2	5.7	2.4	3.0	1.6	3.4				
β-Ureidoisobutyrate	$[M–CH_3]^+$	*m/z* 275	7.8	7.8												
	$[(CH_3)_2Si=N^+HCON=CH_2]$	*m/z* 129	5.9	5.7												

Case 1 was detected in our pilot neonatal screening study. Cases 2–7 were chemically diagnosed as having urea cycle defects by metabolome analysis. Case 1, β-ureidopropionase deficiency; case 2, ornithine transcarbamylase deficiency; case 3, argininosuccinate synthetase deficiency; case 4, argininosuccinate lyase deficiency; case 5, arginase deficiency; cases 6 and 7, carbamoylphosphate synthetase deficiency. Abnormality *n* is expressed based on total creatinine (GC/MS) or creatinine (Enz).

differentiating between this and carbamoylphosphate synthetase deficiency. In general, severe hyperammonemia in the neonatal period favors the latter possibility.

A high concentration of uracil can also be owing to bacterial degradation of pseudouridine, which results in low pseudouridine concentrations. In our pilot study of newborn screening, the abnormality of 7 and −11 for uracil and pseudouridine, respectively, was found in the urine of a neonate with a bacterial infection. Both thymine and orotate were within the normal range. A urine specimen taken one week later confirmed this case was only transient uraciluria, and the levels of both uracil and pseudouridine had modulated to within ±1 SD. Metabolome analysis can greatly reduce the likelihood of a misinterpretation, by simultaneously evaluating a large number of metabolites both markedly increased and decreased.

Of the secondary hyperammonemias, eleven are organic acidemias, eight are lactic acidemias, seven are caused by fatty acid oxidation defects, four by hepatic dysfunction, and there are four other causes, including citrin deficiency. The eleven IEMs termed organic acidemias, which cause secondary hyperammonemia, can be sensitively, accurately, and differentially diagnosed by the simultaneous analysis of metabolites in urine. The secondary hyperammonemias known as lactic acidemias arise from pyruvate dehydrogenase complex disorders, pyruvate carboxylase deficiency, and oxidative phosphorylation disorders caused by abnormalities in electron transport complex I, II, III, IV, and V. These are all the known primary lactic acidemias. Galactosemia and hereditary fructosuria, which cause secondary hyperammonemia, can be screened for by simultaneously evaluating the indicators galactose, galactitol, galactonate, and fructose. Primary lactic acidemia is diagnosed if the blood lactate level is high enough to exceed the capacity of reabsorption by more than 2 mM. In our pilot study of neonatal screening, we identified a neonate with lactic aciduria who was soon found to have an E1α deficiency in the pyruvate dehydrogenase complex. Nonetheless, the methodology needed to diagnose lactic acidemias by urinalysis is not yet complete. Despite this, urine metabolome analysis is helpful for the screening and/or chemical diagnosis of 30 of the conditions that result in hyperammonemia.

2.6. Differential diagnosis of other metabolic disorders

The GC/MS urine metabolome analysis also enables the differential diagnosis of three pyrimidine degradation disorders (dihydropyrimidine dehydrogenase deficiency, dihydropyrimidinase deficiency and β-ureidopropionase deficiency) [22]. Differential diagnosis for conditions in which β-ureidopropionate increases in urine includes β-ureidopropionase deficiency, propionic acidemia (PCCD), methylmalonic acidemia, and six primary hyperammonemias [23]. Recently more rapid tandem mass spectrometric methods have been reported that target uracil, dihydrouracil, and β-ureidopropionate [24], and uracil, thymine, dihydrouracil, dihydrothymine, β-ureidopropionate, β-ureidoisobutyrate, β-alanine, β-aminoisobutyrate, and creatinine, respectively [25].

In Japan, our pilot study of newborn urine screening showed that the incidences of PCCD, methylmalonic acidemia, pyrimidine degradation disorders, and urea cycle disorders including OTC deficiency were higher than that of galactosemia (1/3500) or hyperphenylalaninemia (1/70,000). PCCD and methylmalonic acidemia both had incidence of one per 20,000 neonates [5].

Propionyl-CoA and methylmalonyl-CoA are the catabolic intermediates of several essential amino acids (isoleucine, valine, methionine, and threonine), odd-chain fatty acids, and the cholesterol side chain. Propionyl-CoA is metabolized to D-methylmalonyl-CoA by biotin-dependent propionyl-CoA carboxylase (propionyl-CoA: carbon-dioxide ligase; PCC, EC 6.4.1.3). D-Methylmalonyl-CoA is isomerized to L-methylmalonyl-CoA by L-methylmalonyl-CoA epimerase (EC 5.1.99.1) and then converted to succinyl-CoA by L-methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carbonylmutase, EC 5.4.99.2). PCCD is caused by a propionyl-CoA carboxylase deficiency, and methylmalonic acidemia is caused by a defect in the conversion of methylmalonyl-CoA to succinyl-CoA. Recently, it was shown that neurological deterioration occurs in patients with PCCD, even in the absence of ketosis or metabolic acidosis [26–28], and that fatality is not limited to cases with neonatal onset of PCCD but can occur in those with unusually late-onset [29,30]. Therefore, highly sensitive and specific neonatal screening and chemical diagnosis for PCCD is critical.

Neonatal screening for PCCD using urine specimens has not been performed extensively. Methylcitrate, first described in 1972 [31], is the most reliable indicator for PCCD, although it can also increase in methylmalonic acidemia, and multiple carboxylase deficiency caused by either holocarboxylase synthetase deficiency or biotinidase deficiency, and biotin deficiency. The differential diagnosis of disorders with elevated methylcitrate depends on the presence or absence of methylmalonate, 3-hydroxyisovalerate, or 3-methylcrotonylglycine, which can be determined simultaneously. Urease-pretreatment without fractionation and stable isotope dilution using D_3-methylcitrate allows the satisfactory recovery and sensitive and quantitative analysis of the highly polar methylcitrate. Thus, this method permits the highly sensitive identification of presymptomatic newborns with PCCD.

Two neonates with PCCD, one detected in our pilot study of neonatal screening [5], and one during a search for the etiology of cardiomyopathy, showed the same, distinctly elevated methylcitrate level (70 mmol/mol creatinine), but other metabolites were not increased. The neonates received a chemical diagnosis of PCCD. Their residual PCCD activity was 6.8% of control and they were homozygous for the Y435C mutation in the PCCB gene [32]. The Y435C mutation was identified in the pilot study of neonatal screening in Japan and has proved to be very frequent in Japanese. Follow-up studies suggest that these patients can be treated with dietary restrictions of propionyl-CoA precursor amino acids and carnitine supplementation. We continue to monitor the levels of methylcitrate as well as amino acids that are precursors of propionyl-CoA in the urine of these patients by metabolome analysis.

In methylmalonic acidemia, there are four known etiologies, two caused by an apomutase deficiency (mut^0, mut^-) and two caused by defective adenosylcobalamine synthesis (cblA and cblB) [33]. Recently, methylmalonic acidemia due to methylmalonyl-CoA epimerase deficiency was described [34]. Another inborn error in cobalamine metabolism causes combined methylmalonic acidemia and homocystinuria and is detectable by the urease-pretreatment procedure [21]. Homocystinuria types I and II can be chemically diagnosed. More than 22 etiologies underlie the IEMs of methylmalonate and/or sulfur-containing amino acids, cobalamine and/or folate, including abnormalities of absorption, transport, and metabolism of cobalamine and folate. The administration of cobalamine, folate, or vitamin B_6 causes dramatic improvement in many of these conditions. The important indicators are homocystine, methionine, cystathionine, orotate, methylmalonate, and methylcitrate. Stable isotope-labeled homocystine, orotate, and methionine are used as the internal standards. Further investigations to evaluate the sensitivity, specificity – and hence accuracy – of these chemical diagnoses are in progress.

3. Simultaneous screening and chemical diagnosis of more than 130 IEMs

The simplified diagnostic procedure uses the urease-pretreatment of urine or eluates from urine dried on filter paper, with partial adoption of stable isotope dilution and GC/MS targets more than 200 components, from lactate to homocystine, with a 15-min GC/MS measurement. Capillary GC allows the most efficient separation of metabolites and gives chromatographic information indicating very precise retention times for compounds of endogenous and exogenous origin. Mass spectrometry combined with GC, with full-scan mass spectra and automated identification, quantification, or semiquantification can be used to evaluate 200 metabolites. After correction for creatinine or total creatinine (creatinine plus creatine) and $\log_{10}$ transformation, the values were compared with those obtained from age-matched healthy control groups. An abnormality n, in terms of the mean above $n \times$ SD, is obtained automatically to evaluate the target metabolites. For each target, more than two ions are chosen because of the complexity of the metabolome[35].

This procedure, technically practical yet comprehensive from the metabolic point of view, is a valuable tool for the screening and diagnosis of many diseases. One can screen for and make a chemical diagnosis of more than 130 IEMs, including branched chain amino acids (22 etiologies), primary hyperammonemias (8 etiologies), aromatic amino acids (17 etiologies), pyrimidines and purines (9 etiologies) [36–40]. Galactose [2,41], glucose and fructose can also be targets as well as their oxidized and reduced metabolites. Neuroblastoma, 17 primary lactic acidemias, and 5 fatty acid oxidation disorders can now be detected. The metabolome-based diagnosis and screening for each etiology is, in general, successful. For the diagnosis of only a few diseases, especially in the differential diagnosis of primary lactic acidurias and fatty acid oxidation defects, does the sensitivity of detection in urine need to be improved and the criteria for differential diagnosis completed.

IEMs have been successfully diagnosed using GC/MS, if the targets or disease markers for the IEM in urine are volatile or become volatile by derivatization. However, if the targets are purine or pyrimidine nucleotides, purine nucleosides, or acylcarnitines, such IEMs cannot be diagnosed solely by GC–MS but require the complementary use of soft ionization MS. Very thermally unstable or nonvolatile amino acids or acylcarnitines, cannot be analyzed by GC–MS but can be quantified by soft ionization MS. Such amino acids include homocitrulline, argininosuccinate, and arginine, indicator for HHH syndrome, argininosuccinate lyase deficiency, and arginase deficiency, respectively. For long-chain fatty acid oxidation defects, long-chain acylcarnitine is the target, but it is not excreted in urine. Accordingly, the long-chain fatty acid oxidation disorders cannot be screened for or diagnosed by urinalysis. The short- and medium-chain-length acylcarnitines are excreted in urine. Therefore, disorders of short- and medium-chain fatty acid oxidation can be diagnosed by soft ionization MS using urine as the test material. Their screening or chemical diagnosis can be done by GC/MS using targets, such as acylglycines, dicarboxylates, and other organic acids.

4. Summary and conclusion

Human urine contains numerous metabolites and can provide the needed evidence for successful screening or chemical diagnosis of metabolic disorders. The bottom-up approach from metabolome analysis to enzyme assay, protein analysis, and genetic evaluation is feasible and cost-effective. Metabolite analysis can be used to evaluate enzyme function. In addition, if the metabolites are quantified and evaluated, the near-comprehensive detection of genetic mutations is possible. That is, almost all mutations that lead to a significant reduction in enzyme activity can be detected, even ones that are uncommon or unknown. Insufficient levels of coenzymes or activators, defects in post-translational modification, or abnormal subcellular localization can also affect metabolite levels. Many steps, including the absorption or transport of coenzyme precursors and their conversion to coenzymes, which are controlled by proteins and altered by mutations in their genes, are also reflected in the metabolome with various degrees of clarity. Therefore, the very sensitive and quantitative GC/MS metabolome analysis of urine is nearly comprehensive for detecting mutant genotypes, and can be used to screen simultaneously for a large number of such mutations. In fact, this metabolome procedure may eventually permit simultaneous screening for numerous mutations in human genome.

The metabolome analysis is effective for detecting abnormal metabolic and/or nutritional conditions in patients, including acquired vitamin deficiencies, the biological response to drugs, or excess nutrient loading. We described two cases of mild phenylketonuria caused secondary. This procedure is also useful for monitoring and evaluating treatments for patients and animals, including liver transplantation and gene therapy. This metabolome approach is noninvasive, valid, feasible, and appli-

cable for patients with many IEMs, regardless of the presence or absence of clinical symptoms.

In the near future, the number of diagnosable IEMs will increase, resulting in more opportunities to detect abnormalities in the human genome. Metabolome profiling may in the future allow individuals to obtain comprehensive genetic information to use for their own personalized medicine over their lifetime.

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