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Original Article

Metabolic products in urine of preterm infants characterized via gas chromatography-mass spectrometry

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Abstract: Objective: To characterize the metabolic products of urine associated with preterm birth, thus providing clinical guidelines for intestinal and parenteral nutrition in preterm infants. Methods: Urine samples of 47 preterm infants and 45 full-term infants were collected and prepared for trimethylsilylation by treatment with urease. The levels of lysine, phenylalanine, histidine, ornithine, fumaric acid, malic acid, succinic acid, lactose, stearic acid, and 4-hydroxyphenylacetic acid were detected by gas chromatography-mass spectrometry (GC/MS), and statistically analyzed. Results: The normalized concentrations of the following metabolites in preterm infant urine samples were significantly lower than that of full-term infant urine samples: lysine (P = 0.003), phenylalanine (P = 0.001), histidine (P = 0.006), ornithine (P = 0.000), fumaric acid (P = 0.002), malic acid (P = 0.006), succinic acid (P = 0.000), lactose (P = 0.000), stearic acid (P = 0.000) and 4-hydroxyphenylacetic acid (P = 0.000). Conclusions: The results of the GC/MS analysis indicated that amino acid, carbohydrate, and fatty acid metabolism defects exist in preterm infants. The use of GC/MS to determine metabolic products in urine samples could be helpful for prospectively evaluating the nutritional status of preterm infants, and therefore providing clinical guidelines on reasonable nutritional support.

Keywords: Preterm infant, full-term infant, gas chromatography-mass spectrometry, urease treatment, metabolic product

Introduction

Preterm birth, defined as birth before 37 completed weeks or 259 days of gestation, is the second leading direct cause of deaths in children younger than 5 years [1, 2]. In 2010, the preterm birth rate worldwide ranged from about 5% in European countries to 18% in African countries [2]. The United States is one of the top 10 countries in numbers of preterm births, and the Institute of Medicine estimated that 12% of live births are premature. These account for close to 60% of the total spent on initial neonatal care [3]. In China, 7.8% of neonates are preterm [4]. This rate has risen steadily in recent years, perhaps due to increased numbers of abortions, multiple births, premature rupture of membranes, or gestational hypertension [4, 5].

Premature birth is associated with a significant risk of neonatal mortality and morbidity due to low gestational age and birth weight and underdeveloped organ systems, especially in infants delivered before 32 weeks [6]. However, preterm birth also increases the likelihood of health and developmental problems in the long term, including neurodevelopmental disabilities, lung and gastrointestinal problems, and vision and hearing loss in early childhood [6, 7]. In addition, when preterm survivors reach school age, hidden disabilities in learning and behavioral problems become apparent and persist into adolescence; rates of cardiovascular

and metabolic disorders tend to be higher in middle age and beyond [8-10].

Most babies who are small for their gestational age catch up to normal in weight during the postnatal period [11]. This accelerated growth pattern in infants of low birth weight lowers the incidence of respiratory disease, diarrhea, and infant mortality [12], and has a positive effect on neurodevelopment [13]. Nevertheless, a significant number of studies have linked accelerated weight gain in early growth with subsequent risks of obesity in later life [11, 14-16], and an increased risk of chronic disease in adulthood [17, 18]. Hence, efforts to promote growth in infants who are small for gestational age should consider the possible short- and long-term benefits and disadvantages. To achieve a healthy catch-up in growth, nutritional support requires careful monitoring to minimize adverse long-term effects on metabolism and body composition, while ensuring adequate growth.

Metabolomics is the study of all the metabolites, in a biological cell, tissue, organ, or organism under a particular set of conditions, that is, a biochemical profile [19, 20]. For non-invasive monitoring of the metabolome of an organism, urine is frequently used. Gas chromatography/mass spectrometry (GC/MS) is a valuable tool for determining metabolites in urine, even in minor concentrations, and has been widely used in mass-screening tests of neonates for earlier diagnosis and treatment of inborn errors of metabolism [21-23].

Shoemaker et al. [24] invented the urease method of sample preparation for GC/MS measurement of intermediary metabolites. In this method, crystalline urease enzyme is used to remove urea from body fluids so that, after dehydration, other water-soluble metabolites may be derived for analysis via gas chromatography.

In the present study, in an effort toward developing clinical guidelines for intestinal and parenteral nutrition in preterm infants, we characterized the metabolic products of urine associated with preterm birth. Specifically, we used a modification of Shoemaker et al.'s protocol [23] to evaluate the levels of lysine, phenylalanine, histidine, ornithine, fumaric acid, malic acid, succinic acid, lactose, stearic acid, and 4-hydroxyphenylacetic acid (HPA) in preterm infant urine samples, relative to those of full-term infants.

Materials and methods

The Medical Ethics Committee of Sixth Affiliated Hospital of Sun Yat-Sen University approved this study. The participants' family members provided their written informed consent to participate.

Equipment and reagents

The gas chromatograph/mass spectrometer was purchased from JEOL (JMS-Q1000GC UltraQuad, Akishima, Tokyo, Japan). The metal capillary column was from Frontier Laboratories (ultra ALLOY, UA5-30M-0.25F Koriyama, Fukushima, Japan). The test tube concentrator was purchased from TAITEC (TC-8F, Koshigaya-shi, Saitama-ken, Japan). The sample concentrator (023-119A) and dry block heaters (L-129A) were from Beijing Laiheng Lab Equipment (Beijing, China). The circulating water vacuum pump was obtained from Shanghai Yarong Biochemical Instrument Factory (SHZ-IIID; Shanghai, China). The microcentrifuge was obtained from Eppendorf (5417C, Hamburg, Germany). The urease (type CIII), bis (trimethylsily) trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS; 99:1), heptadecanoic acid, and creatinine were purchased from Sigma-Aldrich (St. Louis, MO, USA). High-purity nitrogen was obtained from Beijing Oxygen Plant (Beijing, China).The infant formulas Nestle Pre NAN and NAN HA 1 Gold were obtained from Nestle (Vevey, Switzerland).

Study subjects

The study group included 92 newborns, comprising 47 preterm and 45 full-term infants. Both groups were selected from neonates who were delivered at the Department of Obstetrics and Gynecology, Sixth Affiliated Hospital of Sun Yat-sen University from October 2010 to November 2013.

As criteria for inclusion in the study, the gestational ages of the preterm babies were 28-36 weeks and the full-term babies were 37-41 weeks. None of the infants of either group had a medical history of fetal distress, birth asphyxia, or neonatal complications within the first 6

Table 1. Nutrient content and energy density of the Nestle infant formulas, per 100 g

	Pre NAN	NAN HA 1 Gold
Energy density	2084 kJ	2145 kJ
Proteins	14.4 g	9.75 g
Carbohydrates	53.2 g	59.9 g
Fat	25.9 g	26.0 g

postnatal hours; the Apgar score of the neonate was ≥ 8; and blood gas and lactic acid tests yielded no abnormal information. None of the participants required or was administered medical treatment such as head box oxygen, continuous positive airway pressure, mechanical ventilation, or antioxidants. None of the mothers of these babies had a medical history of chronic or infectious diseases, malnutrition, or habits such as smoking, drinking, or drug abuse.

The clinical data were collected from the medical records of the newborns and mothers, including gestational age, birth weight, newborn perinatal health status, and mothers' gestational characteristics.

Formula-feeding pattern

All the neonates were fed with infant formula, beginning in the third postnatal hour (**Table 1**). Preterm infants took Nestle Pre NAN formula, 1-2 mL/kg body weight per feeding every 2 hours. Full-term infants took Nestle NAN HA 1 Gold formula, 20-30 mL/kg body weight per feeding every 3 hours. No parenteral feeding was administered to any participants before urine samples were collected.

Sample collection and preparation

A clean urine sample (2 mL) was collected from each participant within the first 24 hours after birth, using a urine collection bag. Urine was either poured into a drying tube, or onto a piece of absorbent filter paper (Matsumoto Institute of Life Science, Kanazawa, Japan). All urine samples were stored at -20°C, and within one week, they were sent to our laboratory for metabolite profile analysis.

To decompose and remove excess urea present in the urine, 100 μL of urine were incubated with 0.04 units of urease at 37°C for 15 min. For accurate quantification, an internal standard solution of 0.2 μg/μL creatinine was prepared in ethanol and added to the urine [25]. Heptadecanoic acid, as an external standard, was also added [26]. Deproteinization was performed by adding 1 mL of ethanol. After centrifugation, the precipitate was removed and the supernatant was evaporated to dryness. Trimethylsilylation of the residue was conducted by adding 100 μL of a mixture of BSTFA and TMCS (99:1, v/v) and heating at 90°C for 40 min. The derived extracts were transferred to microvials under a nitrogen atmosphere, for analysis by GC/MS.

Therefore, creatinine in the original urine sample was quantified relative to an internal standard, as previously described by Shoemaker et al. [25]. The results are presented as: normalized concentration = the evaluation of present metabolite level relative to total creatinine in urine/the upper normal limit of this metabolite in urine.

GC/MS measurement

A JMS-Q1000GC UltraQuad gas chromatograph/mass spectrometer was used for GC/MS measurement. Aliquots (1 μL) of derived extracts were injected into the apparatus using an automatic injection mode. Separation was carried out using an Ultra ALLOY metal capillary column UA5-30M-0.25F. The oven temperature was programmed to increase 17°C/min from 60°C to 220°C, hold at 220°C for 2 min, then increase 15°C/min from 220°C to 325°C, with a final holding at 325°C for 10 min. The temperatures of the injection port, ion source, and transfer line were 260°C, 200°C, and 220°C, respectively. Electron ionization mass spectra were obtained by repetitive scanning at 2.5 cycles/s, from m/z 50 to m/z 650. Helium gas was used as the carrier at a flow-rate of 1 mL/min.

Quality control

We are collaboratively working with Matsumoto Institute of Life Science (MILS), Kanazawa, Japan. The GC/MS data were analyzed and interpreted based on the JEOL GC-MS analysis system (MILS, Kanazawa, Japan) and National Institute of Standards and Technology (NIST) database, and the positive cases were reviewed by experts from both sides. Our laboratory routinely conducts internal quality control and par-

Table 2. Baseline characteristics of the preterm (n = 47) and full-term (n = 45) birth groups

		Preterm	Full-term	*P*
Infants	Gestational age, wk	34.4 ± 1.78 (28-36.86)	40 ± 0.69 (37.43-40.71)	< 0.001
	Birth weight, g	2193 ± 424	3338 ± 213	< 0.001
Mothers	Age, y	31 ± 2.164	32 ± 2.71	0.531
	Gravidity	1.45 ± 0.62	1.4 ± 0.62	0.363
	Parity	1.21 ± 0.41	1.27 ± 0.5	0.573

Table 3. Normalized concentrations of urinary metabolites of the preterm (n = 47) and full-term (n = 45) birth groups

	Preterm	Full-term	*t*	*P*
Lysine	0	0.21 ± 0.28	3.400	0.003
Phenylalanine	0	0.02 ± 0.02	4.064	0.001
Histidine	0	1.24 ± 1.80	3.072	0.006
Succinic acid	0	0.08 ± 0.07	5.336	0.000

ticipates in external quality assessments organized by the European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM).

Statistical analyses

Statistical analyses were conducted using SPSS software (version 13.0; SPSS, Chicago, IL, USA). Concentrations of urinary metabolites in the preterm and full-term groups were first analyzed with a normal distribution test. Normally distributed variables are presented as mean ± standard deviation and differences between the 2 groups were analyzed using the *t*-test. Variables that were not normally distributed are presented as median and mean ranks and differences between the 2 groups were analyzed using the Wilcoxon signed-rank test. A *P*-value < 0.05 was considered statistically significant.

Results

Baseline characteristics

The mean gestational ages of the preterm and full-term birth groups were 34.4 ± 1.78 weeks and 40 ± 0.69 weeks, respectively, a significant difference (t = -19.68, P < 0.001); the mean birth weights were also significantly different, at 2193 ± 424 g and 3338 ± 213 g (t = -16.3, P < 0.001; **Table 2**).

No complication occurred in any of the neonates within the first 6 hours postpartum. Among the 47 preterm infants, after the first 6 postnatal hours, 3 had respiratory distress syndrome, and after the first 24 hours, 10 presented with hyperbilirubinemia. Among the 45 full-term infants, 2 presented with hyperbilirubinemia after the first 24 postnatal hours.

Among the mothers, the mean age, gravidity, and parity of the preterm birth group were 31 ± 2.164 y, 1.45 ± 0.62 y, and 1.21 ± 0.41 y, respectively, and of the full-term birth group were 32 ± 2.71 y, 1.4 ± 0.62 y, and 1.27 ± 0.5 y (**Table 2**). Thus, the 2 groups of mothers were statistically comparable in age, gravidity, and parity (P = 0.531, 0.218, 0.405).

No mother of an infant in the full-term group experienced any pregnancy complication. Among mothers of infants in the preterm group, in the antepartum and intrapartum period, 22 suffered premature rupture of membranes, 15 developed pregnancy-induced hypertension, and 2 had placenta previa.

Metabolic profiling of amino acids in preterm infants

The normalized concentrations of urinary lysine, phenylalanine,and histidine in all infants were distributed normally, and presented as mean ± standard deviation (**Table 3**). The lysine, phenylalanine, and histidine concentrations in all preterm-birth urine samples were below the detectable range. An independent samples *t*-test was used to compare the preterm and full-term birth groups regarding the mean levels of urinary lysine, phenylalanine, and histidine; these were dramatically lower in the preterm infants than the full-term infants (P = 0.003, 0.001, 0.006).

The normalized concentrations of urinary ornithine, fumaric acid, and malic acid of all new-

borns were not normally distributed, and they are presented as median and mean ranks (**Table 4**). The normalized ornithine concentrations in all 47 preterm infants, and 23 of the 45 full-term infants, were lower than the detectable range. Regarding urinary fumaric acid, levels were undetectable in 28 of the 47 preterm infants. Concentrations of malic acid were undetectable in 31 of the 47 preterm infants, and 2 of the 45 full-term infants.

The Wilcoxon signed-rank test was employed to compare the normalized urinary concentrations of ornithine, fumaric acid, and malic acid of the preterm and full-term birth groups; these were markedly lower in the preterm babies than the full-term babies (Z = -4.889, -3.074, -2.721, respectively; and P = 0.000, 0.002, 0.006).

Metabolic profiling of carbohydrate in preterm infants

The normalized concentrations of urinary succinic acid in all infants were distributed normally, and they are displayed as the mean ± standard deviation (**Tables 3**, **4**); those of urinary lactose in all infants were not distributed normally and these are presented as the median. All 47 of the preterm infants, and 2 of the 45 full-term infants, had undetectable levels of urinary succinic acid. Statistical analysis showed that the normalized concentrations of urinary succinic acid and lactose of the preterm birth group were significantly lower than that of the full-term birth group (t = 5.336, P = 0.000 and Z = -6.441, P = 0.000, respectively).

Metabolic profiling of fatty acid in preterm infants

The normalized concentration s of stearic acid and HPA of all urine samples were not normally distributed, and they are presented as the median (**Table 4**). In 7 and 2 of the 47 preterm birth urine samples, stearic acid and HPA levels, respectively, were not detectable. Results of the Wilcoxon signed-rank test showed that the differences in the normalized concentrations of stearic acid and HPA between the preterm and full-term birth groups were significant (**Table 4**, Z = -4.620, P = 0.000 and Z = -4.590, P = 0.000, respectively).

Discussion

In the present study, we used the urease method [24] and analysis via GC/MS to determine the metabolic profile of newborn urine samples, including amino acids, carbohydrates, and fatty acids. To our knowledge, this is the first time that GC/MS has been used to monitor the systemic metabolite pattern of preterm infants. The results showed that the normalized concentrations of urinary lysine, phenylalanine, histidine, ornithine, fumaric acid, malic acid, succinic acid, lactose, stearic acid, and HPA of premature infants were significantly lower than that of full-term infants, and some metabolites were even absent or at levels that could not be detected (**Tables 3** and **4**). Our data is useful for evaluating the nutritional status of preterm infants, and provide valuable nutritional recommendations for preterm infants.

Of the 20 amino acids in the complete range of proteins, 9 are considered nutritionally essential in human beings (leucine, isoleucine, tryptophan, phenylalanine, methionine, lysine, valine, threonine, and histidine) [27, 28], that is, they cannot be synthesized in the body but must be taken in food. Lysine is the primary limiting amino acid for protein synthesis in cereal-based diets consumed by much of the world's population [29, 30]. L-lysine has a major role in calcium absorption, building muscle protein, alleviating pain and inflammation, and facilitating the production of hormones, enzymes, and antibodies. Phenylalanine is a building block of protein, and used as a nutrient supplement for its reputed analgesic and antidepressant effects. Histidine is a proven essential amino acid for infants up to 6 months old, and its omission from the diet may cause a depression in weight gain and nitrogen retention [31]. Ornithine, fumaric acid, and malic acid have crucial roles in the body's amino acid metabolism and energy conservation. Ornithine is a central part of the urea cycle, which is required for arginine biosynthesis [32]. Fumarate is a product of the urea cycle, and an intermediate in the citric acid cycle used by cells to generate energy in the form of adenosine triphosphate (ATP). Fumarate can be converted to S-malate, a salt or ester of malic acid, by fumarase through a carbanion intermediate.

In this study, we found much lower normalized concentrations of lysine, phenylalanine, histidine, ornithine, fumaric acid, and malic acid in the urine of premature infants compared with that of full-term infants. This may be because premature babies have less protein deposition of endogenous amino acids [33], or a lack of

Table 4. Normalized concentrations of urinary metabolites of preterm (n = 47) and full-term (n = 45) birth groups

	Preterm			Full-term			*Z*	*P**
	Range	Median	Mean rank	Range	Median	Mean rank		
Ornithine	0.00	0.00	29.50	0.00-0.06	0.00	44.58	-4.889	0.000
Fumaric acid	0.00-1.56	0.00	29.40	0.04-1.24	0.15	44.80	-3.074	0.002
Malic acid	0.00-5.13	0.00	30.01	0.00-0.86	0.11	43.38	-2.721	0.006
Lactose	0.00-0.42	0.03	24.00	3.23-52.21	10.48	57.50	-6.441	0.000
Stearic acid	0.00-0.48	0.06	26.83	0.02-220.10	10.18	50.85	-4.620	0.000
HPA	0.00-5.82	0.04	26.87	0.03-1.31	0.27	50.75	-4.590	0.000

*Wilcoxon signed-rank test.

metabolic enzymes [34]; Catch-up growth may require more intestinal absorption [35]; There may be less urine excretion of amino acids [36]; or this may be due to the immaturity of kidney function [37].

During early postnatal life, the nutritional support of premature infants is usually dependent upon parenteral nutrition. Commercially available parenteral nutritional formulas provide all 9 essential amino acids and various compositions of nonessential amino acids. However, little research on the comparative efficacy of parenteral formulas of different amino acid compositions has been published in recent years. The measurement procedure used in this study is a valuable tool for further defining optimal doses of total or individual amino acids in parenteral nutrition.

Succinic acid is an important biochemical intermediate generated in a citric acid cycle and eventually becomes energy through the metabolic pathways. In this study, the GC/MS analyses showed that urinary succinic acidin premature infants were below detectable levels. This suggests that the precursors or enzymes for succinic acid production in preterm babies are deficient.

Breast milk has been widely advocated as the best source of nutrition for newborn babies. Lactose is the main carbohydrate in human milk, as well as many other milk-derived products, and accounts for approximately 40% of the total calories provided by breast milk [38]. In newborns, lactose promotes the growth of healthy bacteria and decreases the amount of unhealthy bacteria in the stomach. In our study, the analysis by GC/MS revealed that lactose was surprisingly present in newborn urine, and the normalized levels of urinary lactose in premature babies were markedly lower than that of full-term babies. This may be because lactase activity was reduced in these newborns, and sugar storage in preterm infants was less than that of full-term infants.

In the present study, we found that the urinary lactose levels of individual infants varied and did not follow a normal distribution. Variations in lactose utilization efficiency and postnatal complications in newborn babies may contribute to this outcome. To achieve optimal growth during the neonatal period, the consumption of lactose should be adjusted according to the individual baby's needs, and nutrient intake of glucose should be timely.

Fat is a primary calorie source and begins to deposit under the fetal skin at the 17th week of pregnancy. The fat provides energy and helps maintain body temperature after birth. Human milk also contains fats that are essential for brain development and absorption of fat-soluble nutrients. Long chain fatty acids are needed for brain, retina, and nervous system development, and deposited in the brain during the third trimester of pregnancy. Therefore, supplementation of long chain fatty acids is essential for preterm infants who may not be able to synthesize sufficient amounts to satisfy the needs of brain and retina development. In the present study, the normalized urine concentrations of stearic acid (a long chain saturated fatty acid) in preterm babies were sharply lower than that of full-term babies. Hence, we conclude that dietary long-chain fatty acids should be increased in infant formulas or parenteral nutrition for premature babies. However, catch-up growth is characterized by a disproportionately higher rate of body fat deposition relative to

lean tissue, and catch-up fat early in postnatal life has been intimately associated with hyperinsulinemia [39, 40]. To achieve healthy catch-up growth, dietary fats in nutritional support should be regulated with extreme care, to minimize adverse effects on long-term metabolism, while ensuring adequate growth.

Organic acidemias are a group of inheritable genetic metabolic disorders characterized by the excretion of non-amino organic acids in urine [36]. Most organic acidemias result from a defect in amino acid catabolism, in which an essential enzyme is absent or malfunctioning. Characteristics presentations of organic acidemias include general malaise, vomiting, poor feeding, breathing problems, hypotonia, and spasticity [41-44]. Early detection and treatment can greatly alleviate these effects. The first-line diagnosis of organic acidemias is through urine organic acid analysis using GC/MS. Hereditary tyrosinemia type 1 is caused by a deficiency in fumarylacetoacetate hydrolase, characterized byelevated urine concentrations of HPA, 4-hydroxyphenylpyruvic acid, and para hydroxyphenyllactic acid [45, 46]. In the present study, none of the neonates showed signs of tyrosinemia type 1, and only a few preterm infants had a relatively higher level of HPA in their urine. However, a newborn with an organic academia usually appears healthy at birth and could have a normal urinary organic acid profile for the first few days of life [36]. To avoid misdiagnosis, other quantitative methods and confirmatory testing should be performed for final diagnosis.

Altogether, urease pretreatment combined with GC/MS is an effective method for monitoring the nutritional status of preterm infants, and would be helpful for timely adjustment of the premature baby's nutrient intake to optimize catch-up growth.

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Disclosure of conflict of interest

None.

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