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Original Research

GC-MS and metabolomics analysis of amino acids, glucose and urinary metabolic pathways and characteristics in children with spleen-deficiency diarrhea

Shan Xu[1,2], Shouchuan Wang[1,2*]

[1] Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, 210029, China
[2] Nanjing University of Chinese Medicine, Institute of Pediatrics, Nanjing University of Chinese Medicine, Nanjing, 210023, China

*Correspondence to: wangshiuchaun555@163.com

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Abstract: Diarrhea is a disease, and patients must have bowel movements at least three times per day. This condition may last for several days and may cause dehydration due to fluid loss. Spleen-deficiency makes a person more vulnerable to some infectious diseases. Persistent diarrhea due to spleen-deficiency may affect amino acids and glucose metabolic pathways, and urinary metabolic characteristics. For this purpose, this research was carried out to investigate the pathogenesis and changes of metabolic profiling in urine samples that come from 3 months to 3 years old children with persistent diarrhea due to Spleen-deficiency were analyzed by metabolomics methods based on gas chromatography and mass spectrometry (GC-MS). The urine samples were collected and divided into normal children group (NC group, n=30), persistent diarrhea group (PD group, n=30). The endogenous metabolites in urine were obtained by GC-MS. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) was used to analyze the data. The results were analyzed by one-way analysis of variance and Fold change. Finally, there was a significant difference between the normal group and the diarrhea group, and the significant metabolites, glutamic acid, serine, phenylalanine, histidine, and et al. were identified between two groups. The metabolism of glycine, serine and threonine, arginine and proline, glutathione and pentose phosphate were involved. The result demonstrated that amino acid metabolism and glucose metabolism were the main metabolic pathways and responsible for persistent diarrhea due to Spleen-deficiency.

Key words: Persistent diarrhea in children; Spleen-deficiency; Metabolomics; Gas chromatography-mass spectrometry; Urine.

Introduction

The largest and main cause of diarrhea is infectious agents, which enter the body through various routes. Diarrhea is a common digestive system disease in children, which is listed as one of the four major diseases in pediatrics by the World Health Organization. According to the cause, diarrhea is divided into two categories: infectious diarrhea and non-infectious diarrhea. Various factors cause children's diarrhea to evolve into prolonged and chronic diarrhea. The results of domestic and exotic clinical epidemiological investigations show that prolonged and chronic diarrhea accounts for about 19.0% of infantile diarrhea (1,2). and are mostly seen in infants under one-year-old (3). Prolonged diarrhea leads to nutrient absorption disorders, which then lead to malnutrition, anemia, immunodeficiency, and even hypoproteinemia, growth, and development disorders. Malnutrition, anemia, low immune function cause diarrhea to prolong and affect each other, resulting in a vicious cycle, which is one of the main factors affecting the growth and development of children, malnutrition, and death. Persistent diarrhea is usually characterized by spleen deficiency in traditional Chinese medicine.
Metabolomics, as an important part of systems biology, is regarded as the "biochemical phenotype" of the whole functional state of the organism, which can represent the response and regulation of the whole functional state of the organism under the stimulation of various external factors in a timely, sensitively and truly. Based on metabolomics technology, domestic and foreign scholars have explored the metabolic status of a variety of digestive tract diseases (4). The difficulty of collecting urine samples is relatively low, which is suitable for the exploration of the characteristic spectrum of clinical diseases in adults and children.
In this study, Gas chromatography-mass spectrometry (GC-MS) was used to analyze and identify the differential metabolites of urine in children with persistent diarrhea due to spleen deficiency, and the possible pathogenesis of persistent diarrhea with spleen deficiency was discussed based on metabolomics.

Materials and Methods

Experiment

Instruments and reagents

Equipped with AS1310 automatic sampler, Trace 1310 gas chromatography, TSQ 8000 mass spectrometer, TG-5MS gas chromatographic column (30m × 0.25 mm, 0.25μm), Speed Vac centrifugal concentrator; Allegra 64R centrifuge (Beckman Coulter, USA); CPA225D electronic balance (Sartorius, Germany).1, 2-^{13}C myristic acid (1g), BSTFA (containing 1% TMCS, 10mL), C8-C40 alkane series standard (1mL), pyridine (100mL) and methoxyamine salt (50mg) were pur-

chased from Sigma. The five standards include histidine, serine, glutamic acid, phenylalanine, and hippuric acid (50-100mg, Sigma).

Sample collection and processing

Diagnostic criteria

According to the 8th edition of Zhu Futang Practical Pediatrics published by people's medical publishing house, the diagnostic criteria for children with persistent diarrhea were formulated (5). There are changes in stool characteristics, such as loose stool, watery stool, mucus stool, or purulent blood stool. The frequency of defecation was more than usual, and the course of the disease was 2 weeks to 2 months.

According to the Guidelines for Diagnosis and Treatment of Diseases of Pediatrics in Traditional Chinese Medicine (ZYYXH/T247~286-2012) published by the China Association of Chinese Medicine in 2012, the diagnostic criteria for diarrhea due to spleen deficiency and diarrhea (6). is as follows: loose stool, light in color and not smelly, diarrhea after frequent eating, yellow complexion, thin body, tired spirit, pale tongue, weak pulse or weak fingerprint.

Inclusion criteria

Children with persistent diarrhea due to spleen deficiency meet the diagnostic criteria, aged from 3 months to 3 years old.

Exclusion criteria

Children with dysentery or cholera; Children with cardiovascular, cerebrovascular, liver, kidney, hematopoietic system and mental, neurological diseases, and other primary diseases.

Sample collection

From January 2015 to June 2017, according to the diagnostic criteria for persistent diarrhea of children with spleen deficiency, 30 cases were collected, including 21 boys and 9 girls. All of them were from the outpatient department of Pediatrics in the Affiliated Hospital of Nanjing University of Chinese medicine. Meanwhile, 30 healthy volunteers, 19 boys, and 11 girls were collected. The clinical data are shown in table 1. There were no statistically significant differences in age, sex, body mass index (BMI), and feeding methods between the two groups, which were comparable ($P > 0.05$). There were statistically significant differences in fecal frequency, fecal color, and fecal characteristics between the two groups on the day of sampling ($P < 0.05$), which was consistent with the characteristics of feces in the children with persistent diarrhea due to spleen-deficiency.

About 2 mL of the middle part of the first fasting urine in the morning was stored at -80℃ to avoid repeated freeze-thaw.

Sample processing

The morning urine sample was melted at 4℃ (or ice) and centrifuged for 10min at 14000r/min. 180 μL of supernatant was taken, 20 μL of urease solution containing 30U was added and incubated at 37℃ for 30min. Then 800 μL of methanol (ice bath, containing 1mg/mL of internal standard 1, 2-^{13}C myristic acid) was added, followed by vortex for 10min and centrifugation for 10min at 4℃ at 12000r/min.

Sample derivation: Add 30μL methoxypyridine solution (15mg/mL) to urine samples, swirl for 1min, oscillate for 1.5h, and mix for 5min; Add 30μL BSTFA (containing 1%TMCS) solution, vortex for 1min, then oscillate for 0.5h (37℃, 600r/min); After standing, 60 μL supernatant was absorbed into the glass tube for GC-MS analysis.

Chromatographic and mass spectrometry conditions

The carrier gas is helium and the flow rate is 1.2mL/min. The shunting mode is adopted and the shunt ratio is 20:1. The inlet temperature is 250℃. Heating procedure: the initial temperature was 60℃, kept for 1min, then increased to 320℃ at 20℃/min, kept for 5min.The injection volume is 1μL. EI source was used: the temperature of the ion source was 280℃, the temperature of the ion transmission line was 250℃, the ionization energy was 70eV, the collection range was m/z50 ~ 500, and the collection time was 3.5 ~ 19.0min.

Table 1. Basic characteristics of the individuals included in the study.

Parameter	Diarrhea group (n=30)	Control group (n=30)	P-value
Gender (male/female)	22/8	21/9	0.774[a]
Age(month)	10.00±6.80	12.00±5.06	0.432[b]
BMI	17.00±0.95	17.26±0.89	0.167[b]
Feeding pattern	Hybrid (16)	Hybrid (15)	0.958[a]
	Breast Mike (5)	Breast Mike (5)	
	bottle-feeding (9)	bottle-feeding (10)	
Number of faeces on the sampling day	5.00±1.20	1.50±0.57	<0.001[b]
Stool color	Yellow-green (24)	Yellow-green (0)	<0.001[a]
	Yellow (6)	Yellow (30)	
Fecal character	Watery (9)	Watery (0)	<0.001[a]
	Mushy (18)	Mushy (16)	
	Mucous (3)	Mucous (0)	
	Pus and blood (0)	Pus and blood (0)	
	Forming (0)	Forming (14)	

Note: Values are given as median ± SD or number of individuals. a, test by chi-square test. b, test by Mann-Whitney U test.

Data processing and analysis

Map information of each sample was collected, and MS-DIAL and NIST databases were used to preprocess the obtained original data. Through Peak extraction, substance identification, and Peak alignment, the 3d matrix data set was obtained. The coordinates were: compound name, retention time, retention index and Peak height extracted according to Peak height. After mTIC normalization and Pareto scaling, Metaboanalyst 4.0 was used for multivariate statistical analysis such as principal component analysis (PCA) and partial least square discrimination (OPLS-DA). Then, performing $log2^x$ transformation on the data, the t-test was conducted after the data basically met the normal distribution. According to the *p-value* and False discovery rate (FDR) value obtained by Fold change (FC) and t-test, different metabolites were screened. When FC > 1.2 and $p < 0.05$, the metabolites were statistically significant. According to the accurate molecular weight and MS/MS results and the comparison with HMDB, METLIN database or standard, the relevant metabolic pathways were analyzed by Metaboanalyst 4.0.

Results and Discussion

GC-MS analysis results

The GC-MS analysis of total ion chromatograms (TIC) in the normal children group and the persistent diarrhea group with spleen deficiency is shown in figure 1. Using the NIST database to analyze the GC-MS data, a total of 23 metabolites were screened, including glutamic acid, histidine, phenylalanine, serine, tryptophan, taurine, and other amino acids, ribosol and other sugars, putrescine and other small molecule metabolites, according to the matching degree, possibility and related compound retention index (RI) identified compounds. The internal standard (1, 2-^{13}C myristic acid) and C8-C40 alkane were used as quality control standards, and the retention index and peak area RSD was controlled within 30%.

Metabolic profile analysis and differential metabolites

MS-dial was used to treat the chromatographic peaks screened in urine, and PCA and OPLS-DA analysis were carried out. According to the OPLS-DA score chart (Figure 2), the difference between the normal control group and the diarrhea group was relatively good, suggesting that there was indeed a metabolic difference in urine between the normal control group and the diarrhea group. Through FC > 1.2 and t-test ($P < 0.05$, FDR<0.26) screening, a total of 23 different metabolites that made major contributions to the score chart differentiation were identified.

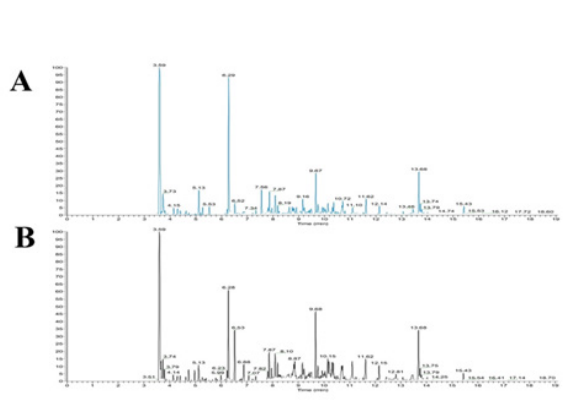

Figure 1. Total ion chromatograms (TICs) of urine samples from (A) diarrhea group and (B) normal control (NC) group.

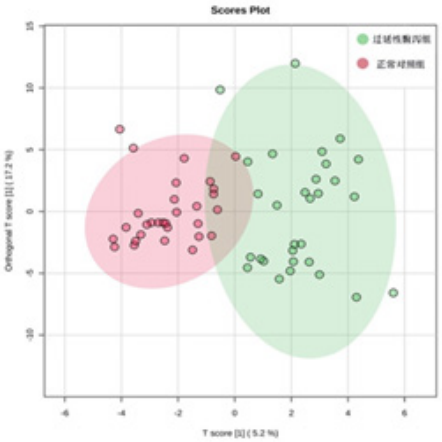

Figure 2. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plots of diarrhea group and normal control group.

Analysis of differential metabolites and their metabolic pathways

MetaboAnalyst4.0 was used for metabolic pathway analysis. According to the pathway influence value and *p*-value, glycine, serine and threonine, Arginine and proline, Glutathione, Pentose phosphate were obtained. There were 5 related metabolic pathways in the biosynthesis of aminoacyl tRNA (enrichment analysis *p*-value < 0.05, Impactor factor > 0.01).

Metabolism of arginine and proline

The arginine and proline metabolic pathways play an important role in maintaining the normal function of the human small intestine (7). Studies have found that arginine can improve gastrointestinal function, accelerate intestinal mucosal regeneration, enhance bacterial clearance and reduce tissue necrosis (8). In this study, D-glutamic acid, guanidine acetic acid, urea and putrescine in the urine of children with persistent diarrhea showed a downward trend, all of the above substances were related products in the metabolism pathway of arginine and proline, suggesting that the disorder of the metabolism pathway of arginine and proline may be one of the reasons for the intestinal mucosa regeneration disorder and prolonged diarrhea.

Metabolism of glycine, serine and threonine

Glycine, serine and threonine metabolic pathways realize the mutual transformation of amino acids, and the synthesis of essential amino acids by non-essential amino acids can maintain the normal physiological functions of the human body. Its content is reduced, can cause intestinal mucous membrane metabolism energy is insufficient, repair component is deficient, inhibit the repair of the intestinal mucous membrane and tissue regeneration. In this study, it was found that the levels of guanidine acetic acid, L-serine, and L-tryptophan in the urine of children with persistent diarrhea due to spleen

Table 2. The differential metabolites identified from urine between model group and normal group.

No	Compound	m/z	RI	RT	P-value	FDR	Trend	Fold change
1	5-Hydroxytryptophol	634	778683	11.6	0.026	0.202	↓	2.682
2	trans-Aconitic acid	453	587501	9.22	0.006	0.078	↓	2.589
3	Aminoadipic acid	442	573167	9.07	0.0001	0.014	↓	3.533
4	Cysteinyl-glycine	523	619538	10.03	0.002	0.058	↓	3.369
5	Gluconic acid	571	694407	10.66	0.020	0.179	↓	1.429
6	D-Glutamic acid	390	528609	8.51	0.004	0.064	↓	2.831
7	Guanidoacetic acid	388	510916	8.39	0.004	0.064	↓	5.300
8	Hippuric acid	495	638462	9.78	0.008	0.085	↓	2.340
9	L-Histidine	535	663944	10.24	0.021	0.179	↓	5.893
10	Maleamate	385	502387	8.5	0.005	0.073	↓	2.544
11	m-Cresol	137	272213	5.36	0.002	0.052	↓	23.248
12	Pyroglutamic acid	336	485159	7.97	0.045	0.255	↓	2.999
13	L-Phenylalanine	397	537401	8.59	0.004	0.064	↓	3.612
14	Putrescine	456	588298	9.3	0.007	0.085	↓	1.362
15	Quinolinic acid	450	582684	9.18	0.041	0.255	↓	1.319
16	Ribitol	449	576302	9.2	0.049	0.262	↓	1.693
17	D-Ribose	426	553947	8.93	0.049	0.262	↓	1.520
18	L-Serine	252	395017	6.88	0.019	0.179	↓	5.876
19	L-Sorbose	516	639323	10.03	0.043	0.255	↓	2.702
20	Taurine	425	555862	8.91	0.041	0.255	↓	1.686
21	L-Tryptophan	641	781484	11.64	0.039	0.255	↓	2.066
22	Uracil	246	385872	6.73	0.022	0.182	↓	4.632
23	Urea	186	328823	6.04	0.0007	0.052	↓	6.231

deficiency decreased, and the above substances were all related products in the metabolism pathways of arginine and proline, suggesting that the metabolism of glycine, serine and threonine was disordered. Wu wen et al. found that glycine has the effect of antagonizing endotoxin and reducing inflammation to protect the intestinal immune barrier (9). It is speculated that the glycine metabolism disorder in this study is another reason that leads to the failure of timely repair of the intestinal immune barrier and the prolonged and repeated diarrhea.

Glutathione metabolism

Glutathione (glutathione) is a tripeptide containing the γ-amide bond and the sulfhydryl group. It is composed of glutamate, cysteine and glycine and exists in almost every cell in the body (10). Glutathione helps maintain normal immune system function and has antioxidant and integrated detoxification effects. The decrease of cysteine, glycine, glutamic acid in urine and the lack of glutathione synthesis materials and related metabolites in children with persistent diarrhea due to spleen deficiency suggest that glutathione metabolism is low. This is a good explanation for the reason that children with persistent diarrhea due to spleen deficiency will have low immunity and repeated infection after a long time. These results are consistent with the decrease of antioxidant metabolites in the urine of ulcerative colitis mice observed by Schicho et al (11). Glutathione also participates in the tricarboxylic acid cycle and glucose metabolism in the body and activates a variety of enzymes to promote the metabolism of sugars, fats and proteins. This may be one of the important factors for protein-energy-malnutrition in children with persistent diarrhea due to spleen deficiency.

Mucous membrane damage of the digestive system can lead to abnormal metabolism of 5-hydroxyindoleacetic acid (5-HIAA) in the urine. Moskwa et al. (11) found that urine excretion of 5-HIAA decreased in patients with irritable bowel syndrome, suggesting that the pathogenesis of functional bowel disease is related to 5-HIAA. Harasiuk et al. (12) reported a decrease in the urinary level of 5-HIAA in patients with ulcerative colitis. Consistent with the results of this study, 5-hydroxy-3-indoleacetic acid in the urine of children with persistent diarrhea due to spleen deficiency also decreased compared with normal children. In addition, previous studies of this research group have confirmed the presence of intestinal mucosal injury in mice with persistent diarrhea due to spleen deficiency, and the main lesion is mucosal villous interstitial edema (13).

In addition, the urine of children with diarrhea showed lower levels of phenylalanine, L-tryptophan, D-glutamate, putrescine, hippuric acid, and taurine than normal children. These substances are closely related to the intestinal flora. Phenylalanine is an essential amino acid, and phenyl compounds are mainly produced by the metabolism of intestinal flora (14). Hippuric acid is its downstream metabolite and derived from spoilage produced by microorganisms in the digestive tract. Tryptophan is broken down into indoles by intestinal bacteria through the tryptophan enzyme, mainly by *E. coli* (15). Indole is considered to be a beneficial bacterial signal for intestinal epithelial cells. Therefore, the decrease of the tryptophan level also indicates the impaired intestinal barrier function and the imbalance of flora (16). D-glutamate has been found to be a metabolite of lactoba-

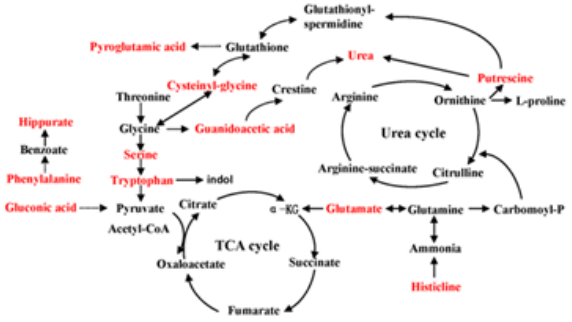

Figure 3. Metabolic pathway network of differential metabolites of two groups. Red color for the significantly down-regulated metabolites in diarrhea groups, black color means undetected metabolites.

cillus (17). Putrescine is found in Citrobacter, Corynebacterium, Cronobacter and enterobacter. Taurine is the growth-promoting factor of bifidobacteria in the human intestinal tract, which optimizes the intestinal bacterial group structure. The above changes in the metabolism of different substances suggest that children with persistent diarrhea due to spleen deficiency have a disorder of intestinal flora. This result is consistent with the clinical research results (18). Recently, by using gas chromatography/flame ionization detector (GC-FID) has been introduced as a convenient, economical, and time-saving method when GC-MS are not available (19, 20). In the future, new technologies such as genome editing and genetic engineering may be the way to go (21).

In this study, GC-MS was used to analyze the changes of urine differential metabolites between children with persistent diarrhea due to spleen deficiency and normal children, and it was found that amino acid differential metabolites, such as glutamate, histidine, phenylalanine, serine, tryptophan, taurine and other substances, decreased significantly in the diarrhea group. Differences in carbohydrate metabolites, such as ribose, were significantly decreased in the diarrhea group. It is involved in the disorders of glycine, serine and threonine metabolism, arginine and proline metabolism, glutathione metabolism and pentose phosphate metabolism pathways. It is speculated that persistent diarrhea due to spleen deficiency causes disorders of amino acid and glucose metabolism in the children and that the damage and repair of the intestinal mucosa and the imbalance of intestinal flora may be the important pathogenesis of persistent diarrhea due to spleen deficiency. The sample source of urine in this study is a non-invasive diagnostic and treatment method, which is easy to obtain and collect. The metabolic differences between normal children and children with persistent diarrhea due to spleen deficiency were evaluated by using the method of urine metabolomics, which can provide a basis for further explanation of the pathogenesis of persistent diarrhea due to spleen deficiency.

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