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Investigation of Levels of Purines and Pyrimidines in Children's Urine

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Abstract

This article presents data on the analysis of purines and pyrimidines by LC/MS. In this paper are given information about the biochemical properties of these compounds and their biological role. The article describes the procedure of the analysis of these compounds by LC/MS with the sample preparation step to obtain a specific result. This article presents reference ranges of normal levels of purines and pyrimidines and changes of these values in various pathologies, such as hereditary diseases in children. This article is intended for clinicians, laboratory diagnostician, and specialists in the field of clinical genetics, pediatric neurologists.

Keywords: Purines; Pyrimidines, LC-MS/MS; Reference ranges

Introduction

Heterocyclic nitrogen bases - purines and pyrimidines - are the initial structural elements of nucleoside and nucleotide molecules. Nucleotides are involved in a variety of biochemical processes. The most known role of purine and pyrimidine nucleotides as precursor monomers in the biosynthesis of RNA and DNA [1]. Exchange of purines and pyrimidines in the body consists of three main ways - synthesis, catabolism and interconversion of nucleosides and nucleotides. Genetically determined defects of enzymes involved in these processes can lead to the development of various metabolic diseases [2]. The symptomatology of hereditary pathologies of purine and pyrimidine metabolism varies widely in severity. Most often with this metabolic disorder, the central nervous system, kidneys and blood system suffer.

The purpose of this study was to investigate the diagnostic significance and establishment of reference ranges of laboratory indicators obtained by the methods of tandem mass spectrometry. The results obtained were first used for a differential diagnosis in patients with hereditary diseases and increasing the effectiveness of targeted treatment.

Materials and Methods

Standards for purines and pyrimidines (Sigma Aldrich, Merck, Fluka (Germany), ammonium acetate, formic acid 99%, methanol for liquid chromatography (Merck, Germany), microfilter for sample filtration (Millipore, USA). The standards for purines and pyrimidines were prepared by dissolution in water, solutions of purine mixtures were prepared by dissolving 5 mM ammonium acetate in solution with 0.1% formic acid.

Patient group

A group of children participating in the study included 42 children suspected of impairing the exchange of purines and pyrimidines. Biomaterial was obtained from patients living in the Moscow region and in the European part of Russia, hospitalized at the NIKI Pediatrics. Yu.E. Veltischeva- Department of Pirogov Russian National Research Medical University (RNRMU).

Sample collection

Fresh urine samples are stored at 4°C and analyzed within one week, for longer storage, urine samples should be stored at -20°C. Before analysis, urine samples in liquid form are centrifuged for 10 min at 10,000 g.

Method for the quantitative determination of purines and pyrimidines in urine by HPLC with mass spectrometry. To determine the purine and pyrimidine bases, the high-performance chromatography-mass spectrometry method was used according to the procedure with modifications [3].

Sample preparation of urine samples

1. To 500 µl of urine, 1.5 ml of 5 mM ammonium acetate was added and the mixture was transferred to a Millipore filter.
2. 200 µl of the filtered sample was transferred to a microplate.
3. 10 µl of the sample was injected into an HPLC/MS-MS system.

Chromatographic conditions

Purine and pyrimidine analysis was carried out using an HPLC system consisting of an Agilent 1200 double gradient pump, a vacuum degasser, and a chromatography column temperature controller (all components of Agilent Technologies, USA) connected to the CTC HTS PAL autosampler.

The reversed-phase chromatographic column Zorbax Eclipse XDB8-C18, 5 µm, 4.0 × 150 mm ("Agilent", USA) was used to separate the analytes. The mobile phase was as follows: 5 mM ammonium acetate was adjusted by volume with 0.1% formic acid (eluent A) and eluent A with methanol in a 9: 1 v/v ratio. All gradient steps were linear and analyzed at a total time of 25 min, including the equilibrating of the chromatographic system.

The detection of the analytes was carried out using an Agilent 6410 QQQ Triple quad tandem mass spectrometer (Agilent, USA). The detector was used in the MS/MS mode in the MRM mode with positive electrospray ionization (ESI), with the exception of orotic acid, which was detected in the negative ESI mode. Nitrogen was used as the

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atomizing gas. Argon and a cell pressure of 0.3 Pa were used as working gas. The initial temperature was set at 300°C and the voltage in the capillary was maintained at 4 kV. The retention times of metabolites and its characteristic ions are shown in Table 1.

Calibration curves were obtained for each analyte by the least squares regression method for each analyte: the ratio of the peak region of the analyte to its concentration in the calibration mixture.

Results

Analysis of urine chromatograms of patients obtained by liquid tandem mass spectrometry from children with various disorders of purine and pyrimidine metabolism in comparison with the urine chromatograms of the control group children - patients with proven absence of hereditary metabolic diseases shows that there are significant differences between them that can be to assess even visually, without quantitative processing of the received data. So, in Figure 1, the mass spectrum of the chromatogram, characteristic of a sample with a normal content of purine and pyrimidine bases, is presented. Figure 2 shows typical chromatograms of xanthine in the urine of a patient with pathology and urine of a patient from the control group.

Based on a retrospective analysis of the urine samples of 369 patients, reference intervals for 19 purine and pyrimidine bases were determined (Table 2). The reference values were defined as the limits corresponding to 2.5-97.5% of the analyte scatter in the sample of patients in which the children were divided into three age groups: 3 months-23 months, 24 months-8 years, 9-17 years.

When analyzing the calculated reference intervals, attention is drawn to the fact that the concentrations of purines and pyrimidines in urine samples are independent of age. It should be noted that only for xanthine and adenine there are insignificant differences (less than 5%) within the limits of reference intervals for the age group 3-23

Name of analyte	Retention time, min	Parent ion (m/z)	Daughter ion (m/z)
Guanosine	16.76	284.2	152.2
Inosine	16.1	269.1	137.1
Deoxyguanosine	18.1	268.2	152.1
Adenosine	21.67	268.2	136.1
Uridine	10.24	245.1	113.0
Citidine	6.51	244.1	112.0
Thymidine	19.01	243.2	127.1
Deoxyuridine	13.49	229.1	113.0
Uric acid	3.6	169.1	96.1
Orotic acid	3.4	157.1	111.0
Guanine	2.7	152.0	135.0
Hydroxymethyluracil	4.89	143.1	82.1
Adenine	6.0	136.1	119.0
Ureidopropionic acid	3.89	133.1	90.1
Thymine	10.7	127.1	110.0
Dihydrouracil	6.8	115.1	73.0
Uracil	7.0	113.1	70.0
Deoxyadenosine	9.78	252.1	136.1
Xanthine	9.1	153.1	110.0

Table 1: Mass parameters (MC) for scrining of purines and pirymidines with retention times for each analytes.

	Age group		
Name of analyte	**3-23 month**	**24 month-8 years**	**9-17 years**
Guanosine	0-1.5	0-1.5	0-1.5
Inosine	0-3.1	0-3.1	0-3.1
Deoxyguanosine	0-2.0	0-2.0	0-2.0
Adenosine	0-2.0	0-2.0	0-2.0
Uridine	0-3.5	0-3.5	0-3.5
Citidine	0-10.0	0-10.0	0-10.0
Thymidine	0-0.2	0-0.2	0-0.2
Deoxyuridine	0-2.0	0-2.0	0-2.0
Uric acid	87-695	87-695	87-695
Orotic acid	0.05-6.0	0.05-6.0	0.05-6.0
Guanine	0-2.8	0-2.8	0-2.8
Hydroxymethyluracil	0-10.0	0-10.0	0-10.0
Adenine	0-2.4	0-2.6	0-2.6
Ureidopropionic acid	0-10.0	0-10.0	0-10.0
Thymine	0-1.7	0-1.7	0-1.7
Dihydrouracil	0-14.0	0-14.0	0-14.0
Uracil	0-25.0	0-25.0	0-25.0
Deoxyadenosine	0-2.0	0-2.0	0-2.0
Xanthine	5.6-29.0	5.9-31.0	5.9-31.0

Table 2: Distribution limits of purines and pyrimidines, meanings of analytes presented in mmol/mol creatinine.

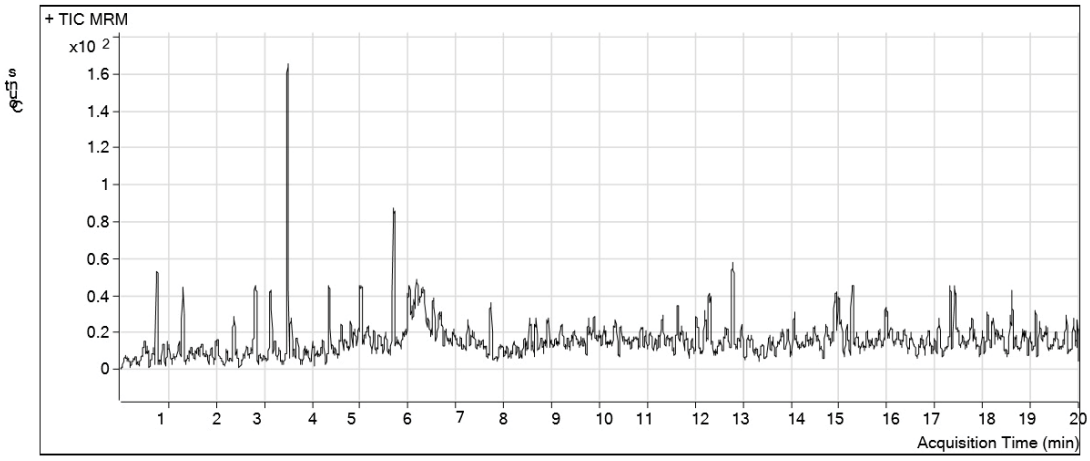

Figure 1: Chromatogram of urine sample with normal concentration of purines and pirymidines. (X-axis – time of chromatography, Y-axis – intensity of signal (in absolute units).

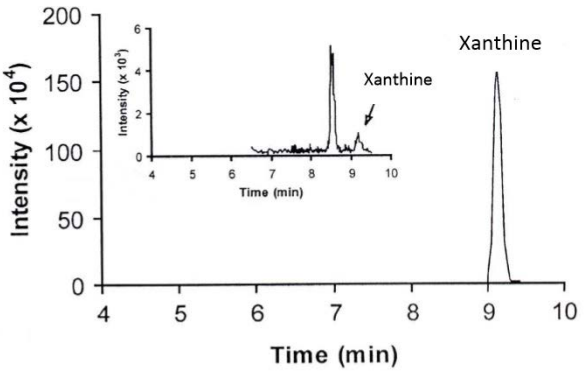

Figure 2: Comparision of typical chromatogram of urine samples of patient with xanthineuria and chromatogram of urine samples with normal concentration of xanthine. (X axis – time of chromatography, Y-axis – intensity of signal (in absolute units).

months and the remaining two age groups. For the remaining purine and pyrimidine bases, no differences in reference intervals between age groups have been identified.

42 samples of children's urine were analyzed, with suspicion of impaired metabolism of purine and pyrimidine bases. In total, 18 children with impaired metabolism of purines and pyrimidines were identified in this group of patients. In all patients, diagnoses were made on the basis of characteristic clinical and laboratory data, as well as the results of molecular genetic studies confirming the presence of pathogenic mutations.

In two cases, a dihydropyrimidine dehydrogenase deficiency was detected in patients; the uracil level in these patients was 325 and 228 mmol/mol of creatinine, which exceeds the reference values by approximately 10 times. The concentrations of thymine in these patients were 25 and 29 mmol/mol of creatinine and exceeded the upper limit of the norm by 12-15 times.

In three cases, the diagnosis of dihydropyrimidinase deficiency was confirmed. The concentrations of dihydrouracil, the main biochemical marker of the disease, in all patients exceeded the upper limit of the norm by more than 10 times. The concentrations of dihydrouralcil in the patient's urine were: 189, 202 and 263 mmol/mol of creatinine. Also, there was an increased content of thymine in the urine of patients, its concentrations were determined in the range of 12-23 mmol/mol of creatinine. Only in two out of three patients with this diagnosis uracil level was exceeded, and in one patient, it slightly exceeded the upper limit of the norm (36 mmol/mol of creatinine) and in the second patient the excess of uracil concentration was more than 3 times as compared with the upper boundary of the reference range (82 mmol/mol of creatinine).

In three cases, the diagnosis of Lesch-Nihan syndrome was confirmed. In the case of the main marker metabolite of this disease - uric acid, determined by HPLC/MS/MS, there is a significant excess of levels of this acid in patients. In children with confirmed diagnosis of Lesch-Nichan syndrome, the level of uric acid was 958, 1325 and 1411 mmol/mol of creatinine. The content of the remaining purines and pyrimidines in these patients were in the normal ranges.

One child had a diagnosis of hyperactivity of type 1 phosphoribosyl pyrophosphate synthase. The concentration of xanthine in the urine of this patient was 64 mmol/mol of creatinine, which is approximately twice as high as the upper reference limit. The levels of the remaining purine and pyrimidine bases in the urine of this patient were within reference values.

Three patients confirmed the diagnosis of hereditary xanthinuria, which is caused by a deficiency of xanthine oxidoreductase. The marker metabolite of this disease is xanthine, its concentrations in the urine of patients exceeded the upper limit of the reference values by 1.4-3 times, and there were 44, 58 and 98 mmol/mol of creatinine, respectively.

In 2 children, a purine-nucleosyl-phosphorylase deficiency was detected. In patients with this pathology, there was a significant increase in several marker metabolites: inosine, guanosine and deoxyguanosine. Concentrations of inosine in urine of these patients were 15 and 29 mmol/mol of creatinine. The levels of guanosine were increased 10-20 times compared to the norm, and there were 23 and 39 mmol/mol of creatinine respectively. There were also increased concentrations in the urine of deoxyguanosine (24 and 26 mmol/mol of creatinine).

In four cases, the diagnosis of hereditary orotic aciduria of the first type was confirmed. In this pathology, patients showed a fairly pronounced increase.

Discussion

When analyzing the literature on the diagnosis of the disturbances in the exchange of purines and pyrimidines, it can be noted that the basic data on the application of the methods for quantitative determination of purines and pyrimidines by the HPLC-MS/MS method were published in the early 2000s with the active introduction of this method into the world scientific and laboratory practice. These sources contain information on the methodology for determining the concentrations of substances [2,3] and, in some articles, various variants of sample preparation of urine bio-samples are given.

When analyzing the literature on the diagnosis of disturbances in the exchange of purines and pyrimidines, it can be noted that these sources contain little information on reference values [3,4]. It was noted that the patient samples studied for calculation of reference values were not formed by age groups. A number of studies have also been published that show the results of quantitative determination of individual characteristic markers in the diagnosis of various congenital abnormalities of purine and pyrimidine metabolism, for example, deficiency of ureidopropionase 1 [5]. The main sources of reference intervals for purines and pyrimidines for the pediatric population are RI ranges without subdivision into age groups. Thus, when comparing the data presented in the book Blau, Duran, Blaskovics [2] and our results, it should be noted that for most reference intervals of analytes are comparable. Only for xanthine, uracil, thymine, dihydrouracil, it is possible to note the difference in the calculated reference ranges, for these analytes, the upper limit is noted to be 1.5-5 times higher than our results. Whereas for orotic acid, the upper limit is about twice as high as our results. It can be noted that the authors of Blau et al. in 2008 [6] published the results of a study of purine and pyrimidine bases in the urine of 104 healthy volunteers aged 2 to 18 years, without separation by age groups. When comparing the authors' data to our results, attention is drawn to the fact that for reference purines and pyrimidines, reference intervals are very close, with the exception of deoxyguanosine and deoxyadenosine, whose ranges were significantly lower than the ranges calculated in our study. The upper limit of the range of orotic acid was also lower in comparison with the RI calculated from our data. It should be noted that in the 2005 publication of these authors, deoxyguanosine and deoxyguanosine were not analyzed.

Disease/marker	Pathological concentrations of markers		
Source	**reference**	**Genetic diseases database Metagene[9]**	**Our results**
Deficiency of dihydropyrimidine dehydrogenase			
Uracil	0-680[2]	50-150	228-325
Thymine	9-440	20-80	25-29
Lesch-Nihan Syndrome			
Uric acid	1800-4400[2]	>1026	938-1411
Hyperactivity of phosphoribosyl pyrophosphate synthase I			
Xanthine	100-120[2]	-	64
Hereditary xanthinuria			
Xanthine	1120-2900[2]	High concentration	58-98
Hereditary orotic aciduria of type I			
Orotic acid	-	1400-5600	380-1540

Table 3: Pathological concentrations of markers of purines and pyrimidines disease [2,7].

Since differences in published results and our study are found for the lowest number of analytes, this is most likely due to the population characteristics of the sample of patients for the determination of RI, the differences due to the methodological features of the quantification methods are unlikely, since the methods of determination are similar and significant differences in methodology not noted [7].

Thus, the differences found in reference intervals determined markers of hereditary metabolic diseases can be related to the features and differences of the analytical process in chromatographic analysis and mass spectrometry. Differences in the sample of patients also contribute to the presence of differences in the analyzed indicators, which once again confirms the need for each laboratory to establish its own reference intervals of markers of hereditary metabolic diseases.

It should also be noted that, for some pathologies, there were no specific values in pathological cases in literature sources, but only information that diagnostic markers in pathology have elevated values include: dihydropyrimidinase deficiency, adenosine deaminase deficiency (Table 3).

Conclusion

Thus, the analysis of literature with the presented values of diagnostic markers of hereditary metabolic diseases in patients with revealed pathologies shows that the pathological values of marker metabolites can also have a significant spread and in the laboratory diagnosis of congenital metabolic disorders, it is necessary to focus on wider ranges of analytes.

References

1. Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, et al. (2001) The metabolic and molecular bases of inherited disease (8th edn). 2: 2528.
2. Simmonds HA (1996) Purine and pyrimidine disorders. In: Blau N, Duran M, Blaskovics ME (eds) Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases. Chapman Hall Medical, London, pp: 341-357.
3. Hartmann S, Okun JG, Schmidt C, Langhans CD, Garbade SF, et al. (2006) Comprehensive detection of disorders of purine and pyrimidine metabolism by HPLC with electrospray ionization tandem mass spectrometry. Clin Chem 52: 1127-1137.
4. Ito T, van Kuilenburg AB, Bootsma AH, Haasnoot AJ, van Cruchten A, et al. (2000) Rapid screening of high-risk patients for disorders of purine and pyrimidine metabolism using HPLC-electrospray tandem mass spectrometry of liquid urine or urine soaked filter paper strips. Clin Chem 46: 445–452.
5. Assmann BE, Van Kuilenburg AB, Distelmaier F, Abeling NG, Rosenbaum T, et al. (2006) E β-Ureidopropionase deficiency presenting with febrile status. Epilepticus Epilepsia 47: 215–217.
6. Blau N, Duran M, Gibson KM, eds. (2008) Laboratory guide to the methods in biochemical genetics. Springer, pp 221-233.
7. http://www.metagene.de/index.html