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AMINO ACIDS PROFILE IN THE DIAGNOSIS OF INBORN ERRORS OF METABOLISM

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REZUMAT

PROFILUL AMINOACIZILOR ÎN DIAGNOSTICUL ERORILOR ÎNNĂSCUTE DE METABOLISM

Introducere: Erorile înnăscute de metabolism (EIM) reprezintă un grup eterogen fenotipic și genetic de tulburări cauzate de defect într-o cale metabolică cu consecințe defectuoase în funcționarea metabolismului și/sau acumularea de metaboliți intermediari toxici la nivelul organismului. În prezent, sunt descrise în jur de 1015 IEM, în timp ce individual sunt rare, incidență estimată cumulativ este de 1:500-800.

Materiale și metode: Cuantificarea aminoacizilor a fost efectuată în plasma a 2 pacienți suspecți pentru o eroare metabolică. Plasma a fost deproteinizată și analizată în vederea cuantificării aminoacizilor prin cromatografie lichidă de înaltă performanță (HPLC) folosind sistemul "Shimadzu LC-20" și "Agilent 1260".

Rezultate și discuții: În rezultatul analizei cromatogramelor obținute au fost identificate concentrații anormal crescute ale unor aminoacizi sugestiv pentru o EIM. În primul caz, aminoacidul fenilalanina avea valori foarte crescute (1064 μmol/L), iar tirozina și aminoacizii concurenți pentru sistemul de transport LNAA (valină, leucină, izoleucină) erau în cantități joase, pacientul facându-se suspect pentru maladia fenilcetonurie (PKU), fiind imediat supus dietoterapiei. Concentrația ridicată de alanină (572 μmol/L) și raportul alanină/lizină crescut de 6,8 a completat profilul investigațiilor metabolice ale pacientului următor cu sugestii importante pentru o eroare metabolică cu implicare mitocondrială. Cuantificarea aminoacizilor la pacienții testați au contribuitt la stabilirea diagnosticului și inițierea terapiei corespunzătoare cu monitorizarea periodica a valorilor aminoacizilor.

Concluzie: Identificarea biomarkerilor din spectrul aminoacizilor prin Cromatografie lichidă de înaltă performanță oferă posibilitatea obținerii unui spectru larg de metaboliți utili în stabilirea diagnosticului sau monitorizarea atât a pacientului acut bolnav cât celui supus unei terapii specifice pentru a evalua eficiența tratamentului.

РЕЗЮМЕ

ПРОФИЛЬ АМИНОКИСЛОТ В ДИАГНОСТИКЕ ВРОЖДЁННЫХ ЗАБОЛЕВАНИЯ ОБМЕНА ВЕЩЕСТВ

Введение: Врождённые заболевания обмена веществ представляют собой гетерогенную группу нарушений, вызванных дефектом метаболического пути с нарушениями метаболизма и/или накоплением токсичных метаболитов в организме. В настоящее время описано около 1015 заболеваний, но кумулятивная частота составляет 1:500-800.

Материалы и методы. Количественный анализ аминокислот проводился в плазме 2 пациентов с подозрением на нарушения метаболизма. Плазму депротеинизировали и анализировали для определения аминокислот с помощью высокоэффективной жидкостной хроматографии с использованием систем «Shimadzu LC-20» и «Agilent 1260».

Результаты и обсуждение: В результате анализа полученных хроматограмм были выявлены аномально высокие концентрации некоторых аминокислот, наводящие на мысль о наличии врождённые заболеваний обмена веществ. В первом случае было выявлена высокая концентрация фенилаланина (1064 мкмоль/л), а тирозин и конкурирующие аминокислоты для транспортной системы LNAA (валин, лейцин, изолейцин)

были в малых количествах, что заставило заподозрть болезнь фенилкетонурию у пациента. Полученные данные определили начало диетотерапии. Высокая концентрация аланина (572 мкмоль/л) и увеличенное соотношение аланин/лизин до 6,8 дополнили профиль метаболических исследований следующего пациента с подозрением на метаболические нарушения с вовлечением митохондрий. Количественное определение аминокислот у испытуемых пациентов способствовало постановке диагноза и началу соответствующей терапии с периодическим мониторингом значений аминокислот.

Заключение: Идентификация биомаркеров в спектре аминокислот с помощью высокоэффективной жидкостной хроматографии даёт возможность получить широкий спектр метаболитов, полезных для установления диагноза или мониторинга как при тяжёлых формах болезни пациентов, так и у пациентов, проходящих специальную терапию, для оценки эффективности лечения.

Introduction. Inborn errors of metabolism (IEM) are a phenotypically and genetically heterogeneous group of disorders that show a dynamic or progressive clinical course and may be associated with a risk of acute metabolic decompensation [1]. They are typically caused by mutations in genomic or mitochondrial DNA, that lead to partial or complete loss of function of an enzyme, cofactor, or auxiliary protein. Inborn errors of metabolism are monogenic disorders that can be inherited in autosomal recessive manner, mitochondrial or, less frequently, in autosomal dominant or X-linked patterns [2].

Currently, around 1015 well-characterized IEMs, causing alterations in specific metabolic pathways, have been described in the literature [3]. IEMs are rare diseases when taken individually, but collectively these disorders are quite frequent in different populations 500 – 800 [4] [5] [6].

In most IEM, clinical symptoms are nonspecific: vomiting, poor feeding, lethargy, hypotonia, seizure, poor linear growth, and poor weight gain. Hyperammonemia is characteristic primarly for urea cycle disorders and therefore it is another strong indication for plasma amino acids analysis. Additional general biochemical parameters of follow up quantitative amino acids analysis are ketosis (high blood and urine ketones), acidosis (blood pH below 7.35) and lactic acidemia (high lactate excretion), alkalosis (blood pH above 7.45), polyuria, polydipsia, and dehydration [4]. At the same time testing of the amino acids are the basis of the IEM diagnosis, especially of intoxication type like amino-acidopathies, urea cycle disorders, some organic acidurias and mitochondrial disorders. The purpose of testing is to either confirm or exclude the diagnosis of a suspected disorder. Once the diagnosis of a particular disorder is established, specific biomarkers are followed to monitor clinical management of the patient.

Materials and methods. We are repporting on two cases of ill children with clinial manifestations specific for intoxication type of IEM in which the amino acid analysis has been performed.

Peripheral blood was drawn by venous puncture on fasting and collected into 5 mL K3EDTA-coated tubes. Then the blood was centrifuged (4500 rpm for 10 min), and the plasma was transferred to 1.5 mL *Eppendorf* tubes and stored at – 80 °C until chromatographic analysis. Before the testing, the protein from plasma were removed by mixing it with acid (trichloroacetic 0,5 mol/L or perchloric 6%). The mix was centrifuged and the extracted supernatant was used for chromatographic analysis. Each probe was filtered through syringe filter, 0,45 µm membrane pore sizes in a 2 ml volume vial or insert and introduced in the amino acid analyzer injector. Plasma amino acids were performed by High Performance Liquid Chromatography within the project «Genomic medicine and metabolomics research in the service of prophylaxis of genetic diseases for healthy generations in the Republic of Moldova» (Acronym: SCREENGEN, Cipher: 20.80009.8007.22) in the Human Molecular Genetic Laboratory (Institute of Mother and Child) by amino acid analyzer - "Shimadzu LC-20" with post-column derivatization with O-pthalaldehyde (OPA) and fluorescence detector and in the laboratory of Scientific Center of Medicine (State University of Medicine and Pharmacy) by amino acid analyzer "Agilent 1260" with UV-VIS detector and automatic injection.

Results and discussion. The chromatograms obtained from the tested patients were suggestive for an inborn error of metabolism in each case.

The first patient, a girl, 5 days old, had a familial history for Phenylketonuria (PKU) of the older brother diagnosed by newborn screening for PKU 4 years ago. Her newborn screening results was abnormal (Phenylalanine >14 mg/dl) (normal <3 mg/dl) being suggestive for disorders of phenylalanine (*Phe*) metabolism. Plasma amino acids analysis was done to confirm abnormal newborn screening results. At the first sight, the obtained chromatogram revealed a great peak, indicating a high concentration of *Phe* (figure 1).

Analyzing the chromatogram, along with high concentration of *Phe* (1064 µmol/L), low level of tyrosine (*Tyr* - 62,7 µmol/L) has been identified. The ratio of *Phe*/*Tyr* = 17 confirmed the disorder of transformation of phenylalanine to tyrosine [7]. Additionally, the levels of the concurrent amino acids for the transport system LNAA (Large Neutral Amino Acids) are in decreased concentrations (valine – 26,6 µmoll/L; isoleucine - 38 µmol/L; leucine – 55,1 µmol/L) because of the occupation of the transport way preferentially by increased level of

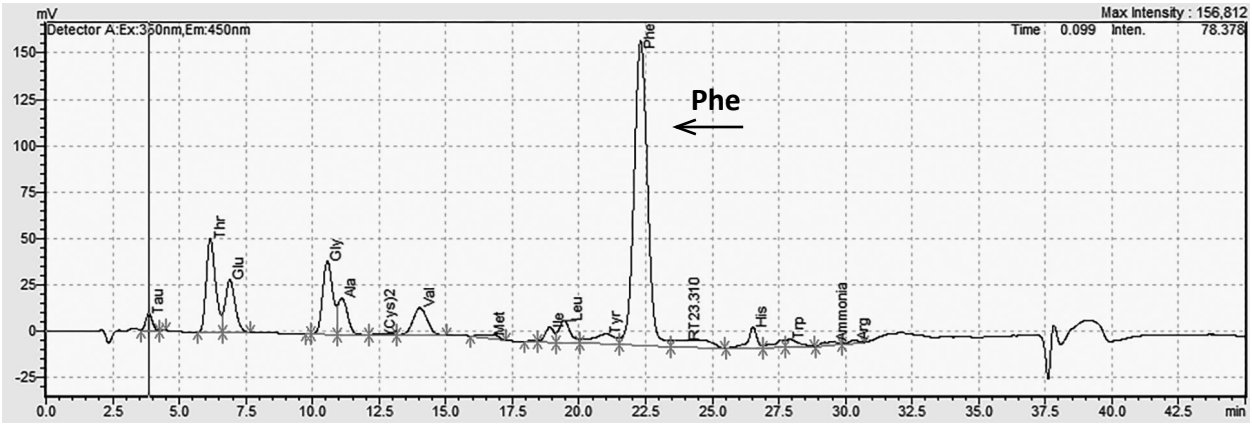

Fig. 1. ***Plasma amino acid chromatogram of the first patient***

Phe. Based on these biochemical results, we suspect PKU which needs confirmation by genetic analysis.

Phenylketonuria is one of the most frequent aminoacidopathies, caused by incapacity of Phenylalanine hydroxylase enzyme to metabolize *Phe* due to mutations in *PAH* gene (around 98% cases) and in rare cases by mutations in genes implicated in biosynthesis of tetrahydrobiopterin.

High level of *Phe* is very toxic for the nervous system development that is why the treatment strategy for PKU consists in reducing the intake of *Phe* from food [8]. The early started diet has a better prognosis for the mental health of the children [9].

In this case, the results from newborn screening confirmed by HPLC analysis were enough to start the diet therapy in order to reduce the probability of mental retardation appearance. The described patient was on low *Phe* diet from the first days of life with systematically monitoring of *Phe* blood level and periodically monitoring of amino acids profile. HPLC analysis was done immediately in order to initiate the therapy as fast as possible.

The second patient was a 2 years old boy. After a free symptoms period of 15 months, the clinical state of the patient got worse, manifested by severe metabolic acidosis crisis with tachypnea, respiratory and feeding disorders, hypotonia, blepharoptosis, lethargy, fatigue, skin allergies, sialorrhea, and partial alopecia.

In order to establish a diagnosis, there has been done a wide metabolic work-up that included LC-MS/MS of DBS samples, urine organic acids and plasma amino acids.

First line investigation showed a variable pH (7,23-7,43), high lactate level (3,1-5,9 mmoll/L), normal ammonia level (34-68 μM/L). Consequently, blood spot sample analyzed using LC-MS/MS revealed high concentration of Propionil-L-Carnitina (C3=3,504 μM/L, (normal<2,7 μM/L), Hidroxiisovaleril-L-Carnitina (C5OH=2,317 μM/L, (normal<0,65 μM/L), and high ratio of C5OH/C0 - 0,076 (normal< 0,035), and C5OH/C8 – 75,274 (normal<13). Additionally, by organic acids analysis in urine has been identified high levels of 3-OH-isovalerianic acid, 3-OH-propionic acid and 2-metilcitric acid in the presence of pyruvic and lactic academia. No ketone bodies have been identified in the urine.

Plasma amino acid profile offered important clues for diagnosis. High concentration of alanine (*Ala* - 572 μmol/L) has been identified and along with the value of the ratio *Ala/Lys* of 6,8 indicated the mitochondrial involvement (figure 2).

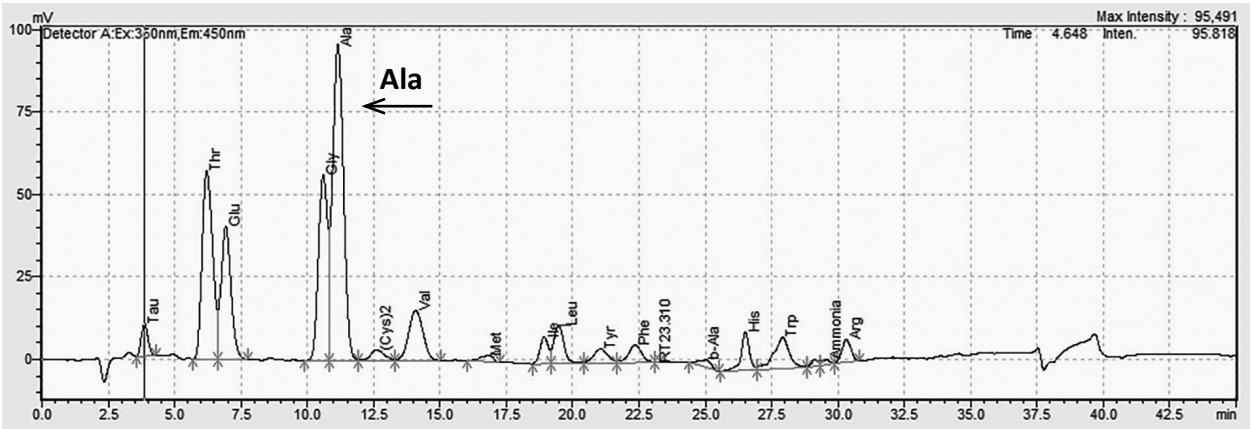

Fig. 2. ***Plasma amino acid chromatograph of the second patient***

Based on clinical manifestation and laboratory data, there is a strong suspicion for a disorder of biotin metabolism - Multiple CoA carboxylase deficiency (MCD). Biotinidase deficiency is an autosomal recessive metabolic disorder in which biotin is not released from proteins in the diet during digestion or from normal protein turnover in the cell. This disorder is well treatable by oral biotin supplementation as a cofactor of carboxylases and treatment should start as soon as the diagnosis is made. In this case, the patient received 20-40 mg biotin per day, without any diet, with evaluation of individual tolerance to the medication by gas analysis and clinical dynamics. The patient was absolutely responsive to the treatment and the clinical state has improved.

Confirmative diagnosis.
It is well known that HPLC analysis of amino acids is an important tool in the IEM diagnosis and it has a large utilisation in advanced specialised laboratory. However, only amino acids profile of the patient can not give complete information for making a correct diagnosis. In order to have a wide view on disease manifestations at the biochemical level, laboratory investigations should be supplemented with data from acylcarnitine profile, urine organic acid profile, mass spectrometry and NMR spectroscopy of body fluids. Confirmation of a suspected diagnosis requires enzymatic assays, biopsy of affected tissues, and genetic tests. Mutational analysis should be done to definitize the diagnosis that allows to patient to start with specific therapies or to have genetic counseling for affected families.

Conclusion.
1. The clinical presentation of metabolic disorders may be non-specific and similar to more common conditions. The initial work-up for metabolic disorders includes quantitative analysis of amino acids that helps to narrow the spectrum of the suspected disorders.
2. The HPLC analysis of plasma amino acids allowed diagnosing the PKU by identification of abnormal levels of *Phe* that was enough to initiate the specific therapy. At the same time plasma amino acids profile fortified the diagnosis of MCD by quantification of high levels of *Ala* amino acid and abnormal value of *Ala/Lys* ratio.
3. Amino acid analysis is an important tool in the diagnostic of different types of IEM and should be used in every uncertain case.
4. Each case should be confirmed by genetic tests.

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