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DIAGNOSIS OF INBORN METABOLIC DISORDERS ASSISTED BY NMR SPECTROSCOPY – RECENT CASES FROM INSTITUTE OF MOTHER AND CHILD CHISINAU

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REZUMAT

DIAGNOSTICUL ERORILOR ÎNNĂSCUTE DE METABOLISM PRIN INTERMEDIUL SPECTROSCOPIEI RMN – CAZURI RECENTE INVESTIGATE IN CADRUL INSTITUTULUI MAMEI ȘI COPILULUI CHIȘINĂU

Lucrarea prezintă un istoric al introducerii spectroscopiei RMN în cercetarea și diagnosticul medical și descrie trei tipuri de diagnostic asistat prin spectroscopie RMN care au fost folosite în ultimii ani la depistarea unor cazuri de boli metabolice rare la Institutul Mamei și Copilului din Chișinău. Este analizat detaliat spectrul RMN al unei probe de urină recoltată de la o persoană din lotul de control și sunt prezentate mai multe regiuni semnificative din spectrele RMN uni- și bidimensionale care au permis identificarea markerilor specifici pentru diagnosticarea și monitorizarea unor cazuri de acidurie metilmalonică, acidurie glutarică de tipul 1 și galactozemie la IMC.

РЕЗЮМЕ

ДИАГНОСТИКА ВРОЖДЕННЫХ МЕТАБОЛИЧЕСКИХ ЗАБОЛЕВАНИЙ С ПОМОЩЬЮ ЯМР-СПЕКТРОСКОПИИ

Данная работа представляет краткую историю введения ЯМР-спектроскопии в медицинские исследования и диагностику, а также три типа диагностики с помощью ЯМР, использованные в последние годы для определения многочисленных случаев редких метаболических заболеваний в Институте Матери и Ребёнка в Кишинёве. Она представляет детальные ЯМР спектры проб мочи обследованных из контрольной группы и многочисленных значимых регионов одно- и двухмерного ЯМР-спектра, которые позволяют идентифицировать специфические маркёры для диагностики и мониторинга метилмалоновой ацидурии, глутаровой ацидурии 1 типа и галактоземии в Институте Матери и Ребёнка.

Introduction

Nuclear magnetic resonance (NMR) spectroscopy has already proven its power in urine analysis. Pioneering works have been carried out by J. K. Nicholson and P. J. Sadler in the mid 1980's once the 400 MHz NMR instruments widely penetrated the chemical community [1-7]. The potential of NMR for diagnosis of diabetes was also realized in the early stages of development of this research field [8,9]. By late 1990's, the NMR urine analysis became an established technique for assisted diagnosis of metabolic disorders. In spite of the great number of published papers on NMR urine analysis, and of the existence of several groups around the world active in the field, there are only a few published results on metabolite ranges determined by NMR. Most of these data are being kept as in-house raw databases. Thus, most of the normal ranges for various metabolite concentrations published to date where obtained by classical methods [10-12]. The reference work for normal values for metabolite concentrations in urine obtained by NMR has been published by the Zuppi's group [13]. The same group also described the comparison of metabolite concentrations for control populations from

two different geographical regions [14]. We also published metabolite concentrations for normal and Diabetes groups from Eastern Europe (Bucharest), and we discussed the results in comparison with previously published data [15-17]. The major advantages of the NMR method are the provision of direct information, and an untargeted global biochemical profile, with minimum sample preparation. In contrast, classical methods require pre-selected conditions for targeted markers. The technique is extremely powerful in tracing abnormal metabolites. However, little has been done until now in terms of establishing databases for healthy individuals. In general, each NMR group is developing its own in-house reference spectral database and uses it to target abnormal cases.

Since 2011 we are using NMR spectroscopy for supporting diagnosis of suspected cases of inborn errors of metabolism (IEM) in Chisinau. The present paper exemplifies NMR assisted diagnoses of rare diseases in the Institute of Mother and Child, Chisinau.

Materials and methods

The urine samples were collected in sterile containers with tight-fitting covers as individual points for each excretion moment. The urine samples were frozen and stored at -20 °C until ^{1}H NMR analysis.

The NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer, using a 5 mm inverse detection multinuclear probe equipped with gradients on the z-axis. The samples were run in 5 mm Wilmad 507 NMR tubes. Before NMR analysis, the samples were allowed to reach room temperature (typically one hour) and centrifuged at 7,000 rpm for 10 min. To 0.9 mL urine, 0.1 mL of a stock solution of 5 mM sodium 3-(trimethylsilyl)-[2,2,3,3-d_4]-1-propionate (TSP) (Aldrich) in KH_2PO_4/KOH/D_2O buffer (Aldrich) was added. The chemical shifts are reported as δ values (ppm) referenced to TSP as internal standard. The ^{1}H NMR spectra were recorded with water presaturation. The pulse sequence used 32 scans, a 90° pulse, 30 s relaxation delay, 3 s CW irradiation and 4 s acquisition time as previously described [15-18].

Results and discussions

Urine is the biological fluid with the highest number of signals visible in the NMR spectrum. This high number of signals on one hand it represents a huge potential for identifying a large number of pathological markers and, on the other hand, it complicates the analysis of the NMR spectrum, which requires special training for interpretation.

If in chromatography based analytical methods (e.g. LC/GS-UV/MS), each metabolite gives one signal in the chromatogram at a well defined retention time, in the NMR spectroscopy there are several signals for the same metabolite. Moreover, each signal may have more than one line, and the position in the spectrum (frequency) of each signal exhibit variations (shifts) depending on the pH of the sample and on the concentration of ions (Ca, Mg, Na). To make things even more complicated, all signals of all metabolites appear in the NMR spectrum in the same time, thus leading to sever overlaps and making the assignments more challenging than in other types of

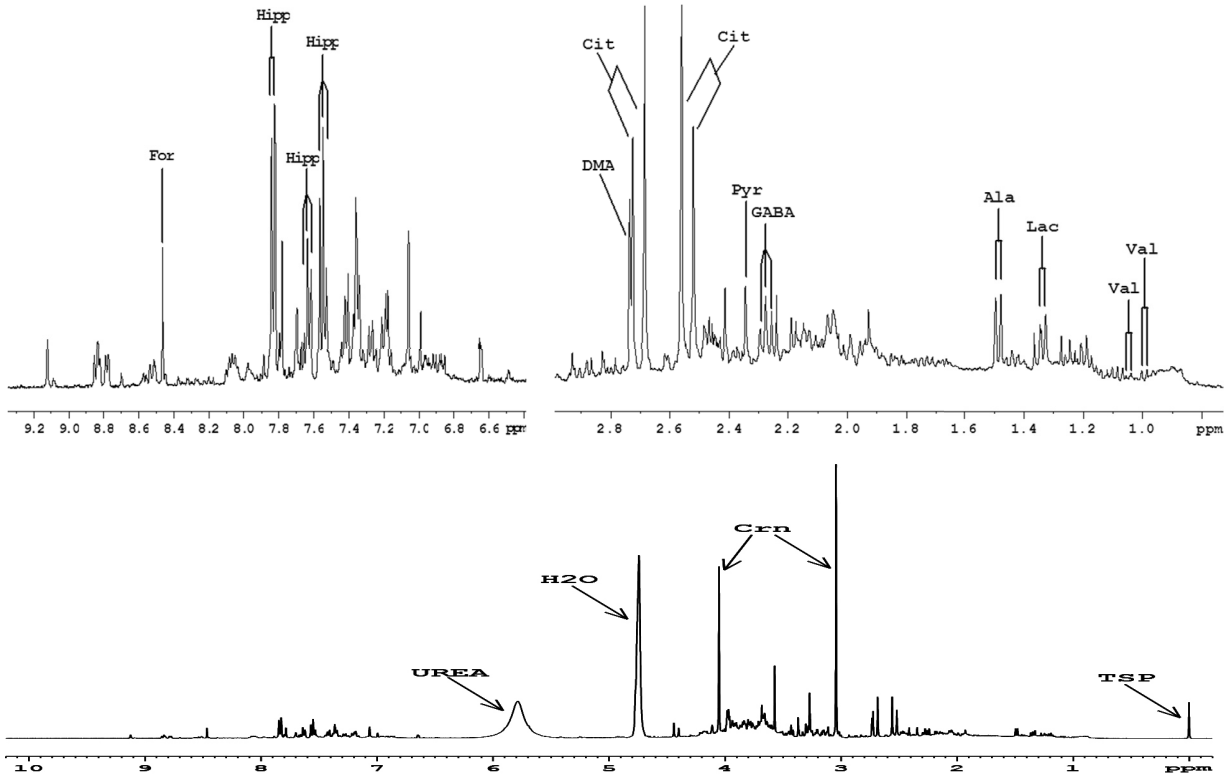

Figure 1. ^{1}H NMR spectrum of a urine sample of a healthy person. Lower trace, the entire spectral window. Upper trace the amplification of the regions 6.5-9.3 ppm (left) and 1.8-2.9 ppm (right).

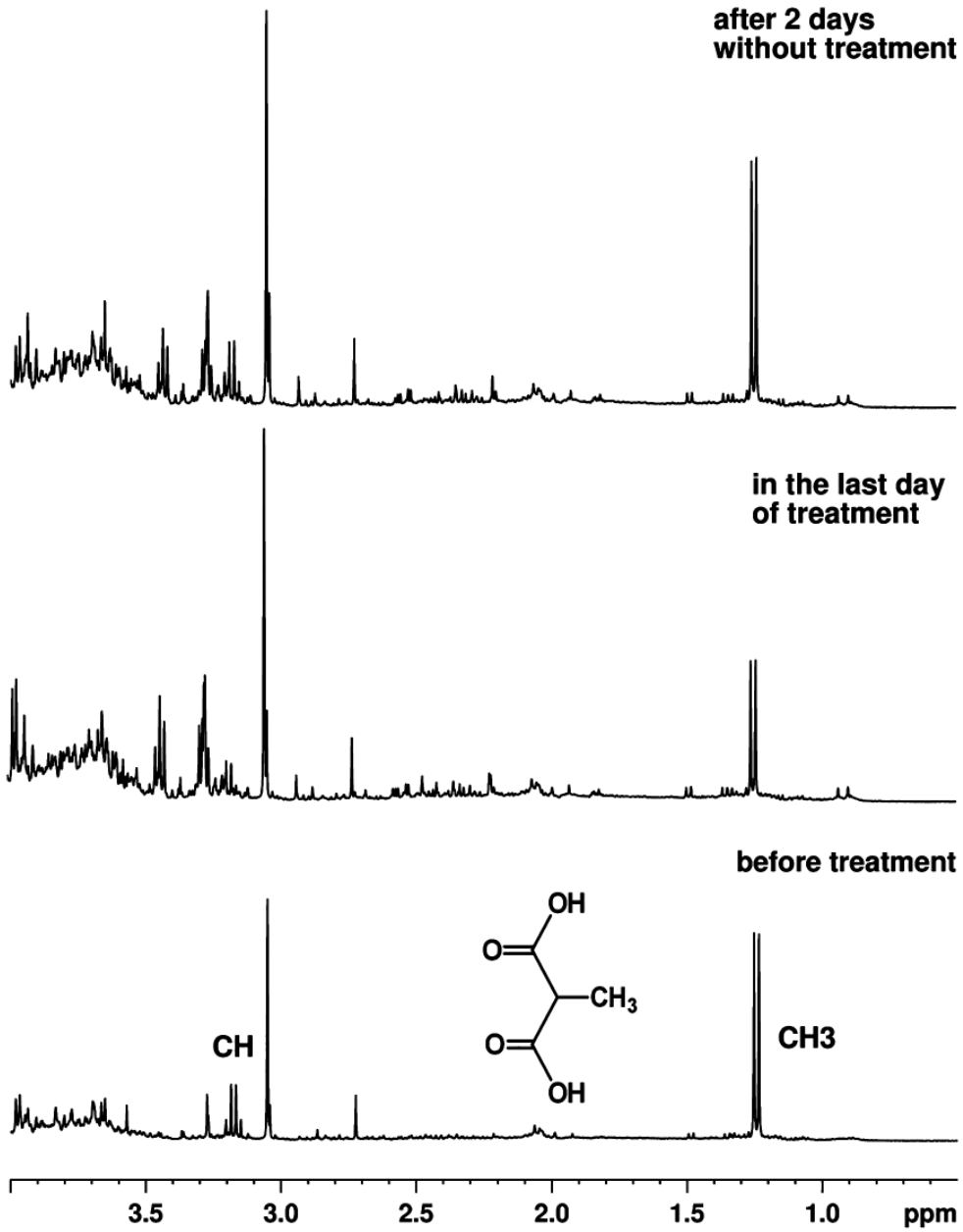

Figure 2. ^{1}H NMR spectra of urine samples from a 2 years old boy with MMA, before and after initiating the treatment.

analyses. Thus, unlike other analytical techniques which have been introduced in clinical laboratories as "black box" automatic analyzers, the NMR spectroscopy involves complex research activities for each analyzed sample. In spite of these complications requiring specialized researchers and higher costs, NMR spectroscopy is nowadays considered one of the most valuable analytical tools for personalized medicine. Also, untargeted NMR is considered the most promising next generation inborn screening technique.

^{1}H NMR spectra of urine from patients with inborn errors of metabolism show in many cases signals associated to markers of the specific diseases. However, there are often cases when the signals of the markers are masked by other signals related to normal metabolites, drugs, specific therapy metabolites, exogenous contaminants, or even food related metabolites. In order to overcome assignment ambiguities additional types of multipulse and two dimensional NMR spectra are often recorded in order to achieve safer diagnosis of inborn errors of metabolism.

Figure 1 presents the whole ^{1}H NMR spectrum of a urine sample from a healthy individual. The figure shows the complexity of such an NMR spectrum.

Below we present several relevant regions of NMR spectra which have been used to identify metabolites associated to various cases of inborn errors of metabolism from the Institute of Mother and Child (IMC), Chisi-

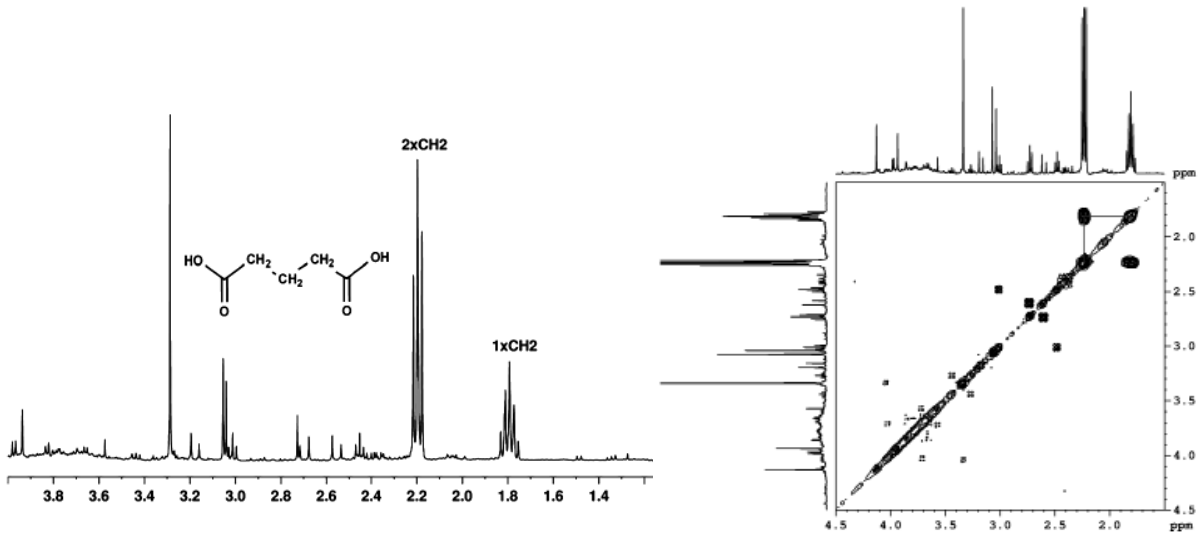

Figure 3. ^{1}H NMR spectrum (left) and the H-H COSY NMR spectrum (right) of the same urine sample from an 18 months old boy with glutaric aciduria.

nau. All selected spectra show well resolved marker signals in order to be self explanatory for the medical diagnosis, avoiding thus the need of presenting extensive additional NMR data which are outside the purpose of this paper.

Methylmalonic aciduria (MMA) is an inherited (autosomal recessive) disorder in which the body is unable to properly process certain proteins and fats (lipids) due to a missing or defective enzyme (methylmalonyl-CoA mutase). The effects of methylmalonic acidemia, which usually appear in early infancy, vary from mild to life-threatening. This condition occurs in an estimated 1:50,000 to 1:100,000 new births. *Figure 2* shows amplification of the relevant region of the ^{1}H NMR spectra of urine samples from a 2 years old boy with MMA. One can see how the effect of the treatment can be successfully monitored by this technique.

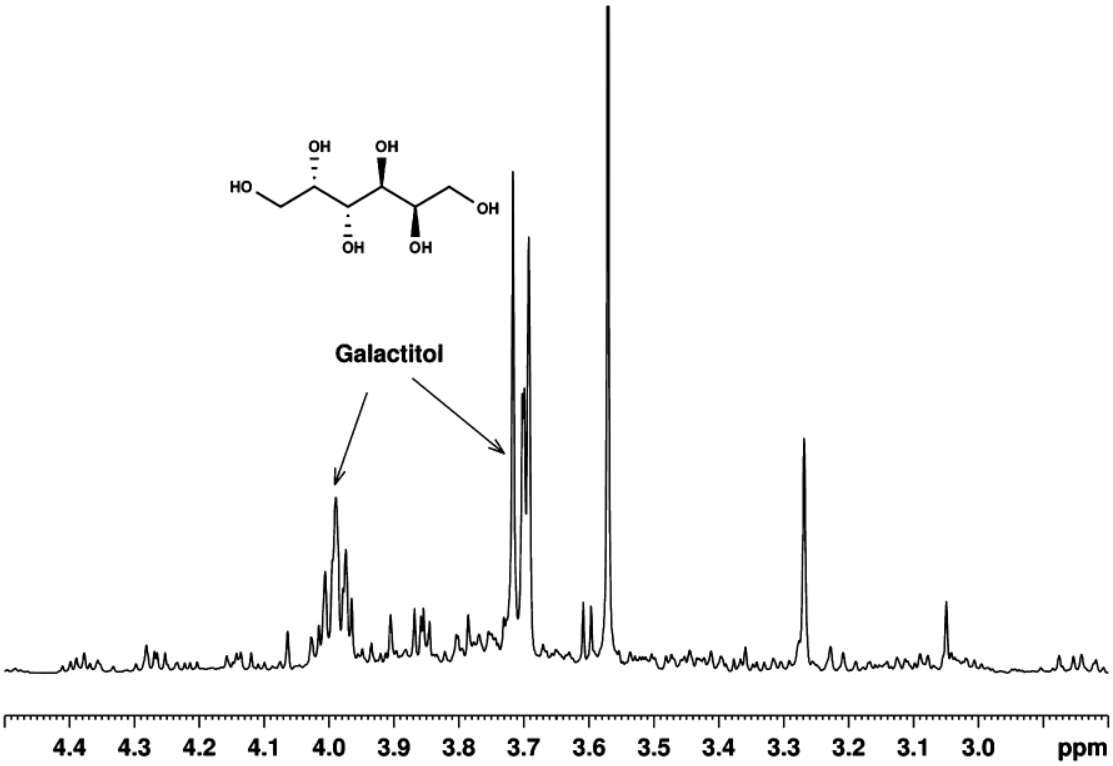

Figure 4. ^{1}H NMR spectrum of a urine sample from a one month old girl with galactosemia monitored during specific diet based on milk without lactose.

Type 1 Glutaric aciduria, an inherited disorder in which the body is unable to properly process certain proteins due to a missing or defective enzyme (glutaryl-CoA dehydrogenase). The condition has an incidence estimated to 1:30,000 up to 1:40,000 new births. *Figure 3* shows the ^{1}H NMR and H-H COSY NMR relevant spectral regions of an 18 months old boy with type 1 glutaric aciduria. The H-H COSY spectrum was used to assign the signals in the ^{1}H NMR spectrum.

Galactosemia is an inherited defect of galactose metabolism caused by one or several enzymes deficiencies (galactose-1-phosphate urydyl transferase for classical Galactosemia) that prevents proper metabolism of galactose. The main dietary source of galactose is lactose, which is the main carbohydrate found in all forms of natural milk. This condition occurs in an estimated 1:60,000 new births. In galactosemia a diet without lactose is mandatory. Before diagnosis, when normal milk is administrated, galactose is building up in organism as the main metabolite of lactose. When the patient is under specific diet, galactose can hardly be detected. However, even when the products based on normal milk are completely excluded, small amounts of galactose are finding their ways into organism, e.g. through fruits and vegetables which are allowed in the diet, or through other metabolic pathways. In the absence of galactose as main marker of galactosemia, in order to indirectly follow up its presence in the organism, we have been monitoring by NMR the presence of galactitol. This compound, a reduction product of galactose, has two characteristic signals: a multiplet in the interval 3.69-3.71 ppm and a triplet centered at 3.98 ppm. *Figure 4* shows the amplification of the relevant ^{1}H NMR spectral region used for monitoring of a one month old girl under specific galactosemia diet.

Conclusions

NMR spectroscopy has the potential for both targeted and untargeted screening of metabolites in biological fluids such as urine. We have presented three types of inborn errors of metabolism for which NMR spectroscopy successfully assisted the diagnosis of several cases of rare metabolic diseases in the IMC Chisinau. The presented cases demonstrate the efficiency of diagnosing rare metabolic diseases when the joint group involves both NMR scientists and pediatricians/geneticists. The described historical cases are the ground basis of a recent expansion of our collaboration to an NMR inborn screening in IMC.

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