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Diagnosis and follow-up of inborn errors of amino acid metabolism: use of proton magnetic resonance of biological fluids

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Summary. Proton magnetic resonance spectra of biological fluids such as urine, plasma and cerebro-spinal fluid can be used for multi-component analysis of highly concentrated species, thus providing information about the general metabolism of the patient. Hydrogen containing analytes in concentration higher than 10 μM are indeed often detectable in biological fluid in 15 minutes by means of an unexpensive 200 MHz spectrometer essentially without sample manipulation. Amino acids, keton bodies, organic acids and other metabolites can be easily estimated by this approach; consequently this technique represents a powerful tool particularly in the diagnosis of inborn errors of amino acid metabolism, when improving the prognosis often depends on a very early diagnosis and on an effective method for monitoring the effects of therapy.

In the present paper, several cases of inherited diseases related to amino acid impaired metabolism will be presented to illustrate the importance in the diagnosis. Phenylketonuria, tyrosinemia, cystinuria, ornithinemia, argininosuccinic aciduria, maple syrup urine disease (MSUD), alkaptonuria, lysinuria and other genetic pathologies were in fact unambiguously and rapidly diagnosed by means of the identification in the biological fluids of the relevant accumulating amino acids and/or of their metabolites. The proposed technique is suitable to become, in the future, a useful routine tool for a wide neonatal screening.

Keywords: Amino acids – Amino acidurias – Organic acidurias – Nuclear magnetic resonance spectroscopy – Neonatal screening – Inherited metabolic diseases

Introduction

Nuclear magnetic resonance (NMR) Spectroscopy is widely known as a very powerful and sophisticated technique enabling the chemist to unravel refined

structural details. For this purpose high magnetic field instruments are necessary. Beside this widespread use, NMR is also suitable for qualitative and often quantitative analysis of many components of several biological fluids, such as urine (Bales et al., 1984), serum (Wevers et al., 1994), cerebrospinal fluid (CSF) (Petroff et al., 1986) and other fluids (Bradamante et al., 1987) provided that the concentration is higher than the sensitivity threshold that depends mainly on the chemical nature of the fluid component. For quantitative analysis one signal is selected among the assigned peaks within the spectrum of the standard analyte. Depending principally on signal multeplicity and on the number of magnetically equivalent hydrogens participating in the signal (Pontoni et al., 1990), sensitivity thresholds roughly ranging from 10 to 200 μM can be estimated if 128 scans of 7 sec each are accumulated (15 min). These thresholds can become sometimes one order of magnitude lower, if the accumulation time can be sufficiently prolonged and if some instrumentation features are adequate. On the other hand, these thresholds can become even higher if interfering components are found in the fluid to be analyzed (Pontoni et al., 1994).

For this reason, a growing body of research concerning proton NMR spectroscopy applications in clinical chemistry has in fact developed over the past ten years because of the above mentioned potential for detection of all

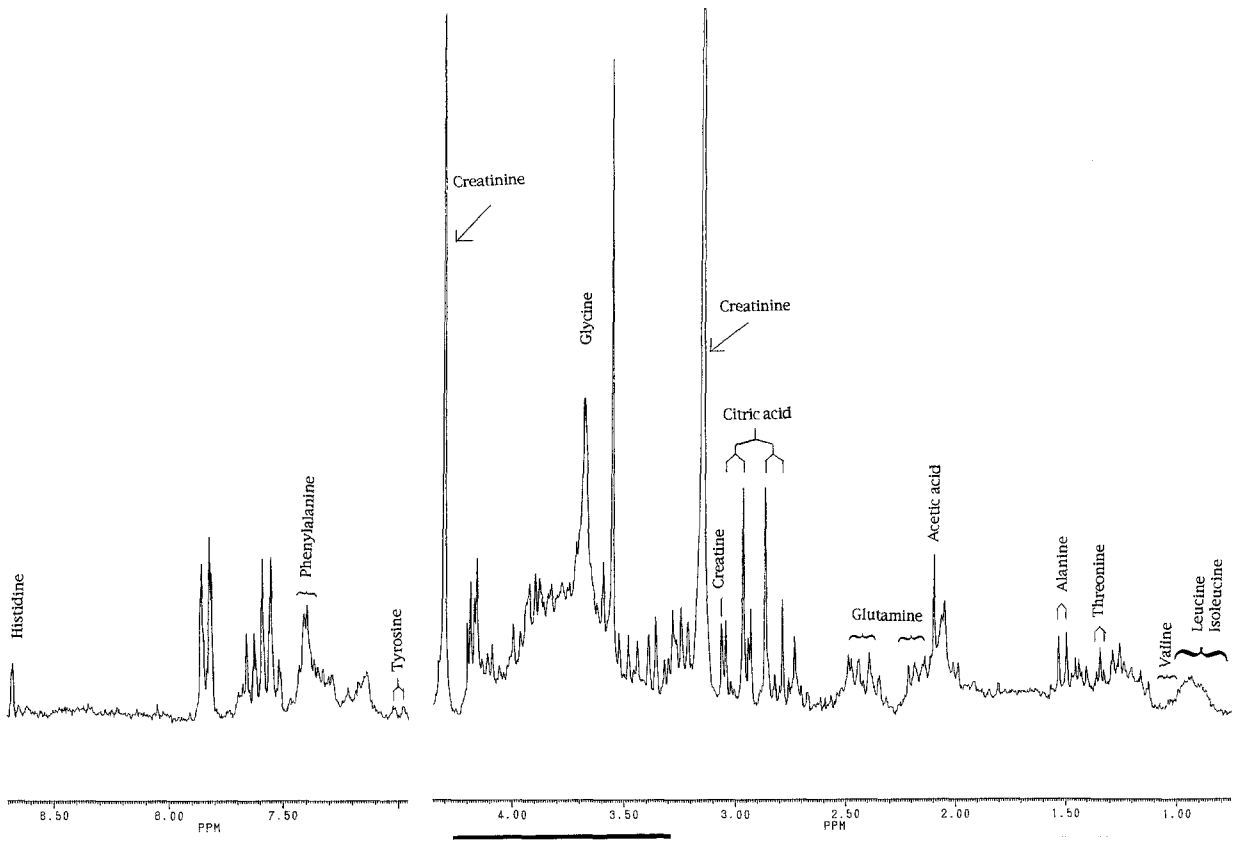

Fig. 1. Urine spectrum of a healthy 8 year old child performed as described under Material and methods. Several amino acids can be identified by means of the most representative peaks

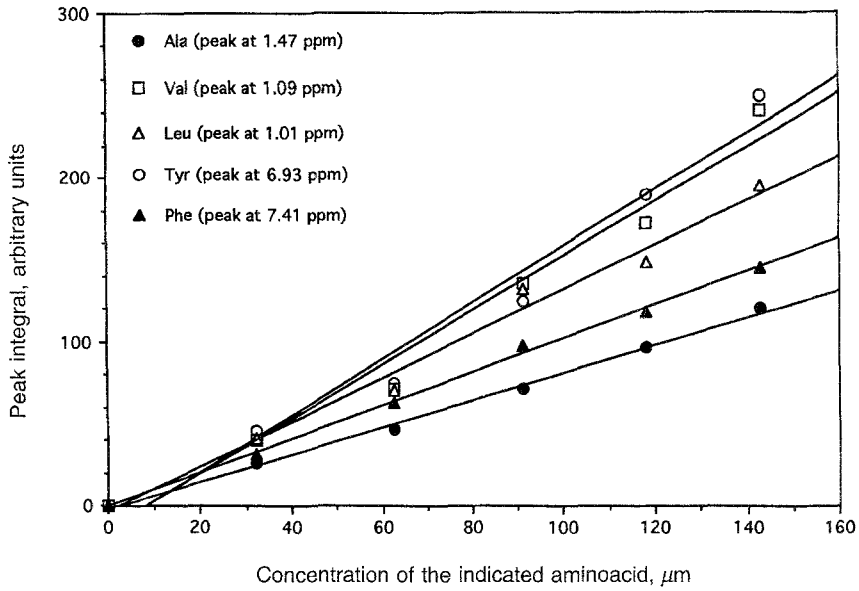

Fig. 2. Linear correlation of integrals of peaks representative of leucine, valine, tyrosine, alanine (in spectra run under the same conditions of urine analysis described under Materials and methods), with respect to the concentration of the aminoacids. Correlation coefficients are always >0.99, indicating the feasibility of quantitative analysis

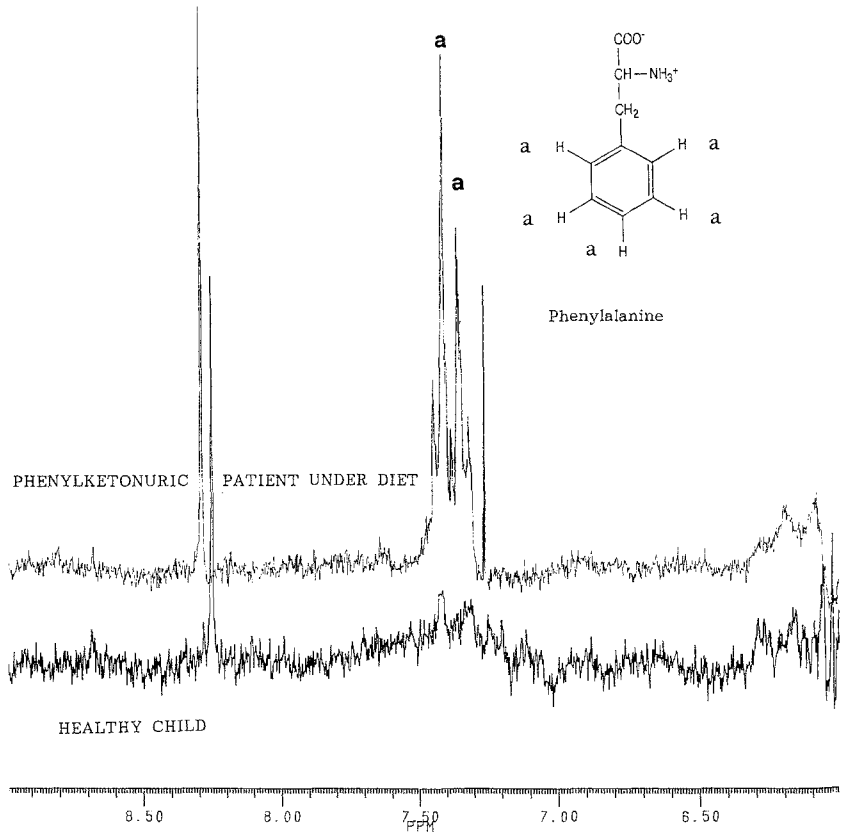

Fig. 3. Urine spectrum of a phenylketonuric patient compared to that of a healthy child of the same age. The peak of phenylalanine overlaps with those of its pathological catabolites phenylpyruvic, phenyllactic and phenylacetic acids, that are typical of phenylketonuria as well, generating a single big signal around 7.41 ppm, whose shape is typical of the pathology. See Material and methods for technical details

proton containing metabolites. Moreover, the technique is rapid, involves no preliminary extraction or derivatization and requires small samples (Lehnert and Hunkler, 1986). Therefore it appears particularly suitable for pediatric clinical care of inborn errors of metabolism (Iles and Chalmers, 1988), when early detection of major metabolic alterations can immediately direct the diagnosis and therapy in order to improve the prognosis.

In the present paper the attention is focused on the qualitative and quantitative analysis of amino acids and of a number of amino acid metabolites that are of diagnostic interest in that they accumulate in inborn metabolic errors as a consequence of blockades in aminoacid metabolism pathways. A small, affordable 200 MHz NMR instrument was selected for the described experiments, in that it was judged to be satisfactoryly adequate, in future, for clinical routine.

Materials and methods

Urine specimens were collected for 24 hours and 300 μL amounts were brought to pH 2.5 $\pm$ 0.03 by means of a Radiometer automatic titration unit equipped with a 2.5 mL automatic burette filled with 3M HCl in order to minimize possible pH dependent slight changes in chemical shift. The sample is then mixed with 100 μL of deuterated water in which a known amount of perdeuterated Sodium trimethylsilylpropionate (TSP) was added; the obtained solution was then inserted into a 5 mm NMR tube and analyzed in a

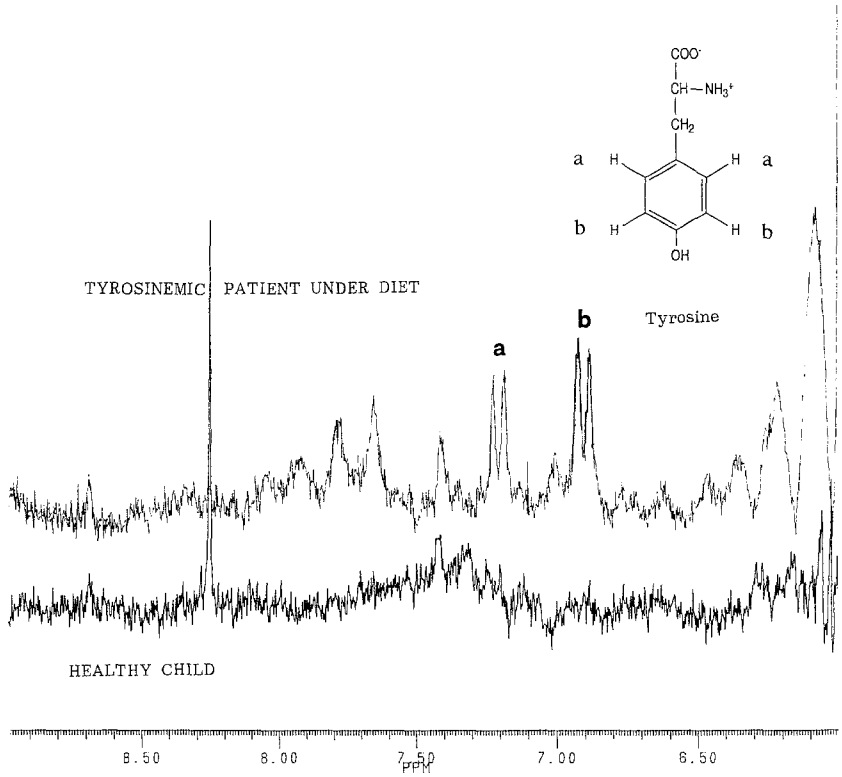

Fig. 4. Urine spectrum of a patient with Tyrosinemia II under low tyrosine diet and of a healthy child. The two doublets of tyrosine ring at 7.23 and 6.93 are characteristic of the disease. See Material and methods for technical details

Bruker 200 AC-E nuclear magnetic resonance spectrometer equipped with a 4.7 Tesla criomagnet with 200 MHz resonance of proton. TSP is taken as a qualitative standard for chemical shift scale as well as a quantitative external standard for peak area calculations according to Tofts and Wray (1988). The peaks indicated in fig 3 to 10 were assigned (Lehnert and Hunkler, 1986) and used for quantitation of the metabolites. Area estimates are performed by the computer of the spectrometer. For routine analyses, 128 and 512 scans of about 7 sec are acquired for urine and deproteinized plasma samples, respectively, although for the mere diagnostic purpose, 32 scans of an urine or serum sample gives already an unambiguous pattern as far as the diagnosis is concerned. The water peak was eliminated by off resonance gate decoupling. All other NMR experimental details are according to Lehnert and Hunkler (1986) with minor modifications.

Results and discussion

The NMR spectra of biological fluids are clearly suitable for qualitative analysis of several amino acids. In Fig. 1 a typical spectrum of urine of a healthy

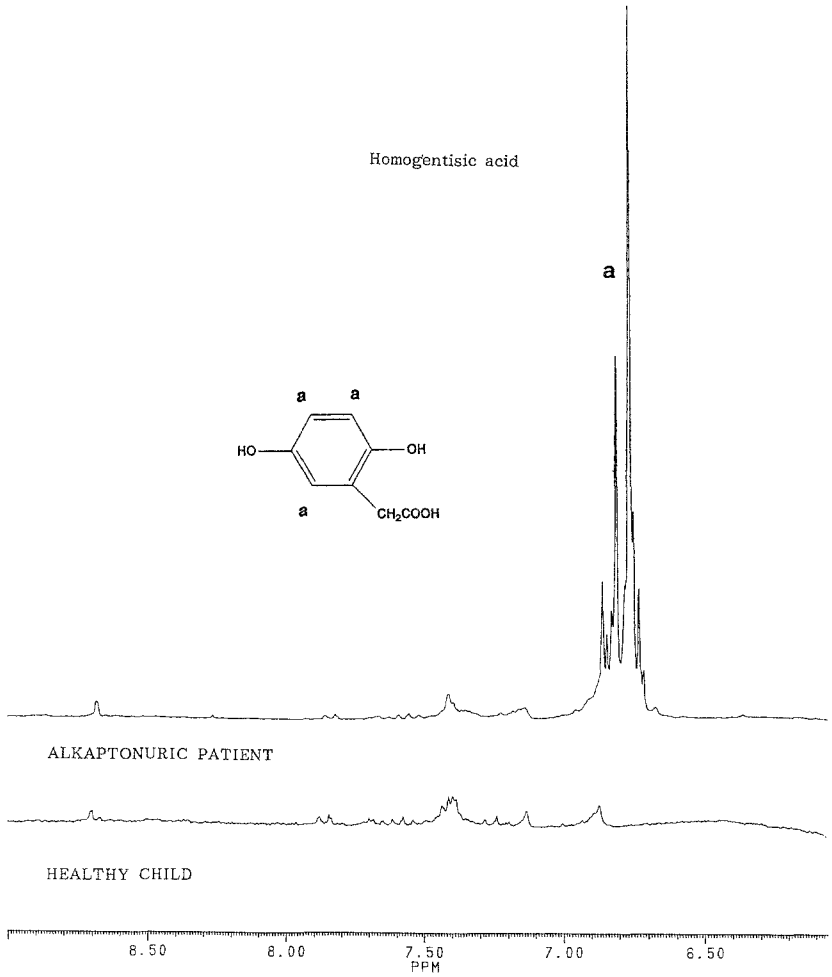

Fig. 5. Urine of an alcaptonuric patient and that of a healthy child. The huge peak is typical of homogentisic acid, that accumulates in this metabolic disorder. See Material and methods for technical details

individual is shown. Several normal urine aminoacidic components, such as valine, alanine, threonine and others are immediately detectable by means of representative peaks. Creatinine is also identified by a signal at 3.14ppm, which is routinely used for measurements of analyte/creatinine ratio. The feasibility of quantitative analysis of some amino acids is illustrated in Fig. 2, that evidences a good linearity of peak integrals related to amino acids accumulating in genetic diseases, as a function of aminoacid concentration (correlation coefficients are always higher than 0.99).

The diagnosis of inherited diseases, which is based on the detection of huge amounts of accumulating metabolites, is unambiguously done by a simple qualitative observation of spectra. Spectra shown in figs 3 to 9 have been performed on urine samples coming from young patients with several among the most common inherited pathologies. These spectra illustrate how the peaks therein have very characteristic shapes that can be easily related to the

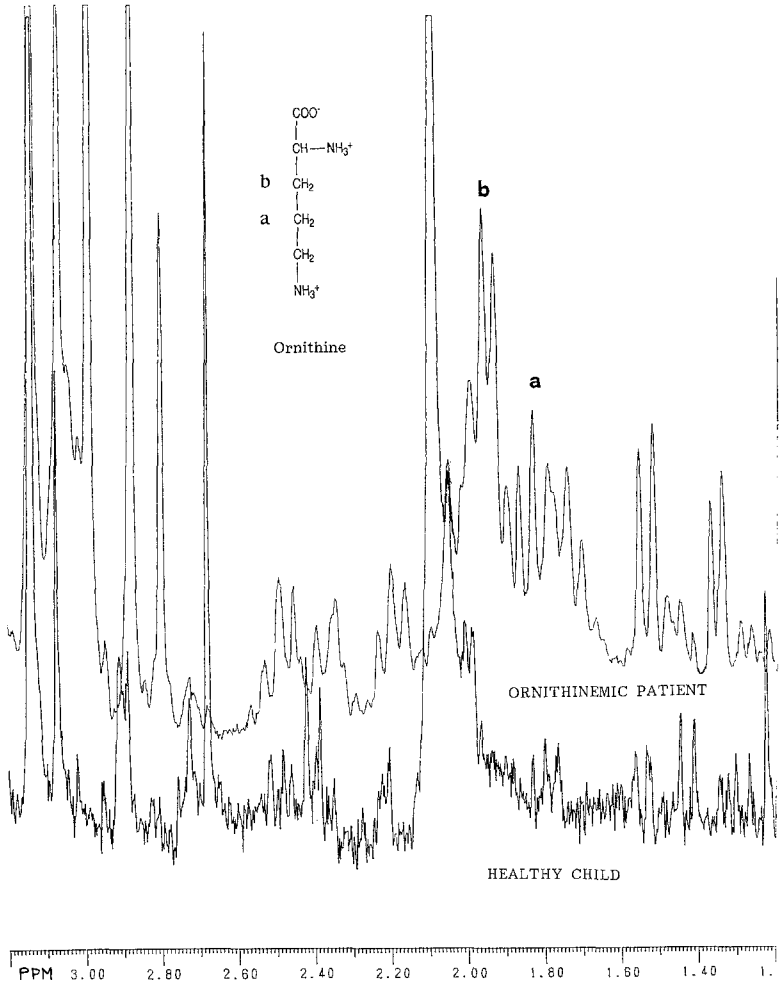

Fig. 6. Urine spectrum of a patient with ornithinemia as opposed to that of a healthy child. Two broad multiplets between 1.7 and 2.1 ppm evidence the high level of ornithine present. See Material and methods for technical details

metabolic disorders, and used as "fingerprints" of the relevant pathologies, thus providing unambiguous information for the diagnosis without requiring quantitative analysis.

Figure 3 illustrates the urine spectrum of a patient with phenylketonuria (a disease caused by impaired phenylalanine hydroxylation) in which a typical, diagnostic signal around 7.41 ppm is representative of accumulating Phe and its derivatives phenylpyruvic, phenyllactic and phenilacetic acids. The diagnosis can be confirmed in the serum spectrum that evidences increased levels of phenylalanine alone. Diet therapy results in normal levels of the pool of serum phenylic metabolites.

Tyrosinemia II, a disorder due to the lack of tyrosine aminotransferase activity is instead identified by the appearance in urine (Fig. 4) and serum spectra of the two doublets of the tyrosine ring (centered at 7.23 and 6.93 ppm) that drop to almost normal levels in serum during low tyrosine diet.

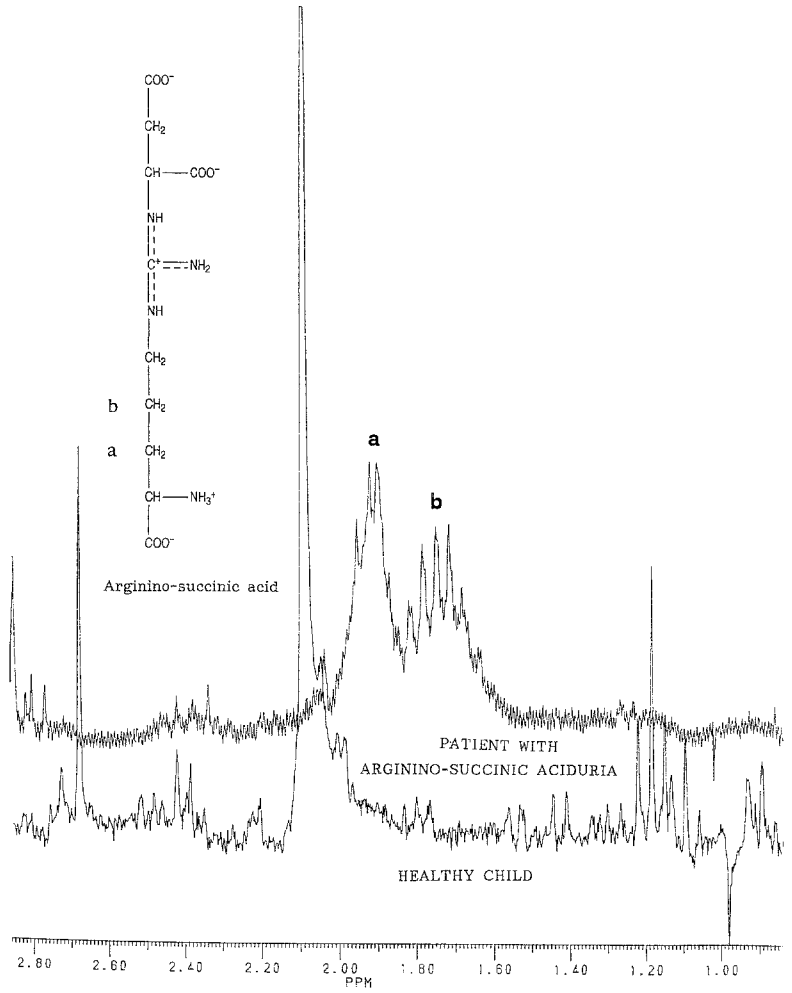

Fig. 7. Urine spectrum of an argininosuccinic aciduric patient together with that of an healthy child. Multiplets at 1.75 and 1.95 ppm are typical of this disorder. See Material and methods for technical details

Another metabolic disorder linked to tyrosine catabolism is known as alkaptonuria, which is characterized by the lack of homogentisic acid oxidase; for this reason, the diphenolic acid accumulates in the urine and can undergo light dependent polymerization, giving a typical dark color to the fluid. As it is illustrated in Fig. 5, homogentisic acid is represented in the urine of an alcaptonuric patient by means of a huge peak around 6.9ppm.

Several inborn errors related to the urea cycle can be diagnosed by NMR urinalysis. Ornithine is clearly detectable in both urine (Fig. 6) and serum spectra of ornithinemic patients, while arginino-succinic acid is present in the urine of a patient with argininosuccinase deficiency (Fig. 7); on the other hand, orotic acid became detectable in a hyperammonemic patient with a deficit of ornithine transcarbamylase (Fig. 8).

Cystinuria, a disorder related to an impaired transport of dibasic aminoacids (see Fig. 9) is usually characterized by accumulation of lysine, arginine, ornithine and cystine. It can be very clearly diagnosed by NMR urine

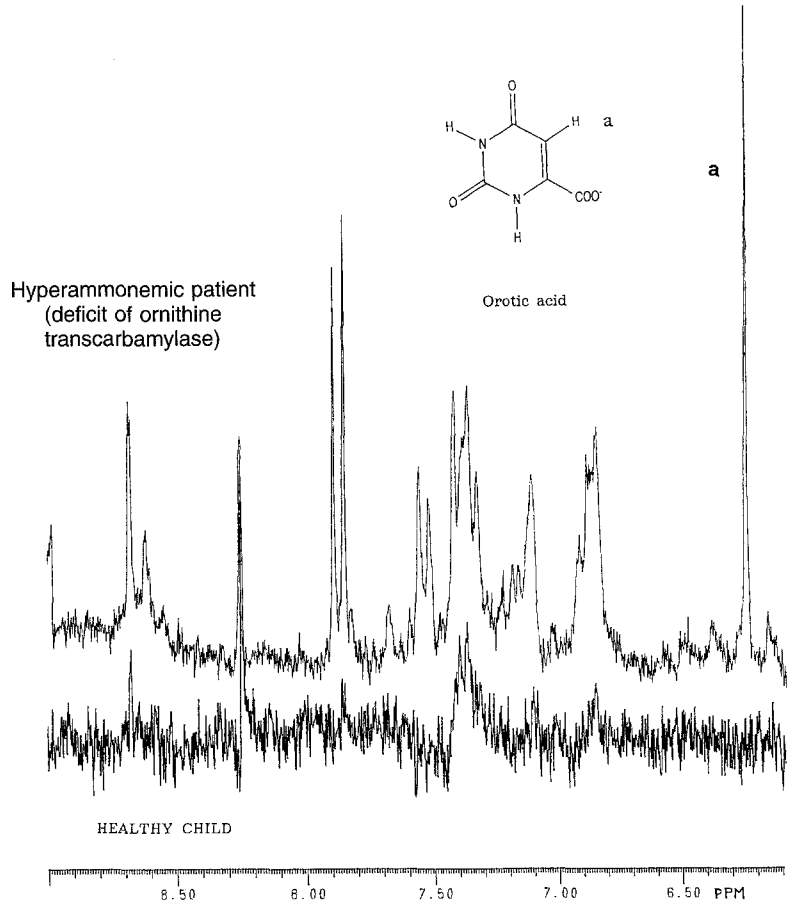

Fig. 8. Urine spectrum of a patient with suspected ornithine transcarbamylase deficiency compared with that of a healthy child. Beside the high levels of glutamine elsewhere in a region not shown of the spectrum, the elevated level of orotic acid, typical of the disease, is clearly detected by means of a singlet at 6.24ppm. See Material and methods for technical details

alysis by means of the three huge multiplets of accumulating lysine between 1.4 and 2.1 ppm. This signal overlaps with the two multiplets of arginine and only partially with those of ornithine; methionine also sometimes accumulates and a very high peak becomes detectable at 2.17 ppm. Cystine itself is also detectable, although in a very crowded spectral region; its presence as a precipitate can be confirmed by NMR analysis of alkalinized urine sediments.

Maple syrup urine disease is generated by the lack of branched chain α-keto acid dehydrogenase activity and produces the accumulation of the three branched chain amino acids leucine, isoleucine and valine, together with their transaminated keto acids 2-ketoisocaproic, 2-keto-3-methylvaleric and 2-ketoisovaleric acids, whose farther degradation generates the typical smell of urine. The NMR signals in urine (Fig. 10) and serum spectra of these metabolites again represent a fingerprint for the pathology and, in order to

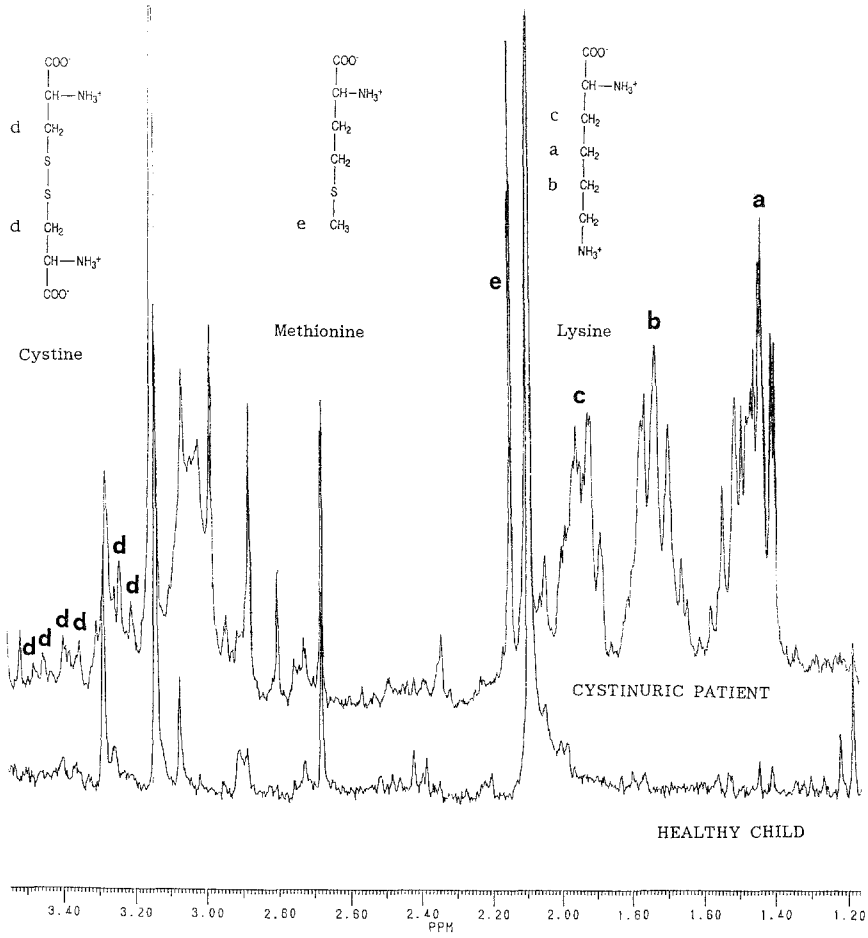

Fig. 9. Urine spectrum of a cystinuric patient compared to that of a healthy child. The most evident anomaly is the presence of millimolar levels of lysine as judged by the three multiplets between 1.4 and 2.1 ppm. Cystine is hardly detectable at 3.23 and 3.27 ppm. Methionine is also detectable by a singlet at 2.17 ppm. See Material and methods for technical details

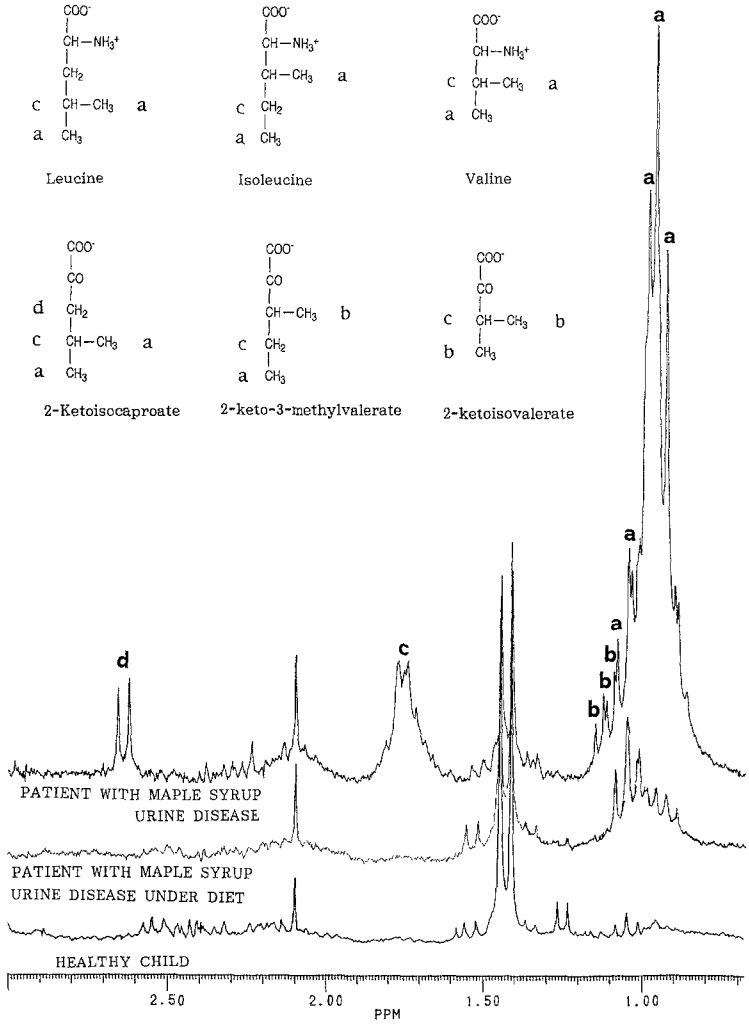

Fig. 10. Urine spectrum of a patient with MSUD versus that of a healthy child. Three amino acid, leucine, isoleucine and valine and three keto acids accumulate in this disorder, giving rise to a very crowded spectrum between 0.8 and 1.2 ppm, which is typical of this pathology. The figure also evidences the effect of branched chain amino acid poor diet in decreasing branched chain amino and keto acids

follow up the effects of dietotherapy, that in this case must be accurate, a quantitative analysis of the accumulated components can be performed by NMR.

Conclusions

The above reported data indicate that proton NMR spectroscopy of biological fluids can become a very potent tool for selective identification of disorders of amino acid metabolism by means of a single quick analysis, with many advantages with respect to well established methodologies such as HPLC, amino acid analysis combined with gaschromatography/mass spectroscopy. No extraction or derivatization is indeed necessary, the original composition

of the fluid is maintained, the analysis is very quick and non destructive, and the results of analysis can be ready in few minutes.

Moreover, although the use of high field instrument for this analysis has some advantages in terms of sensitivity and resolution, we feel that even a small 200 MHz instrument gives sufficient information for an unambiguous diagnosis in all the approached pathologies. Finally, NMR spectroscopy of biological fluids can represent for the therapist a sufficiently rapid tool to monitor the effects of diet even on a daily basis.

The field of inborn metabolic diseases is probably the one in which the use of NMR spectroscopy in the diagnosis as well as in the follow-up, will find in the future more routine application, because of its rapidity, ease of sample manipulation, completeness of metabolite monitoring and number of pathologies that can be screened by a single test. It must also be noted that the price of the described NMR instrumentation does not exceed that of a fully equipped HPLC or of a gas chromatograph/mass spectrometer. For these reasons, NMR spectrometers will become more and more familiar in the hospitals in the years to come.

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