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Diagnosis and follow-up of cystinuria: Use of proton magnetic resonance spectroscopy

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Summary. Proton Nuclear Magnetic Resonance (NMR) Spectroscopy of urine (as well as of other biological fluids) is a very powerful technique enabling multi-component analysis useful in both diagnosis and follow-up of a wide range of inherited metabolic diseases. Among these pathologies, cystinuria is characterised by accumulation in urine of four dibasic amino acids, namely lysine, arginine, ornithine and cystine; the last one, being only slightly water soluble, generates urolithiasis. The mentioned aminoacids can be detected in the urine NMR spectrum of cystinuric patients, the most abundant being the lysine (5 mM and over are often detected), whose typical signals become very high; arginine and ornithine are also usually detectable, although pathologic concentrations are lower (usually below 2 mM).
The proposed NMR technique is also suitable in monitoring the therapy with α-mercaptopropionylglycine (MPG), providing quantitation of several metabolites of interest in the follow-up of the pathology, like cystine, creatinine and citrate.

Keywords: Amino acids – Cystine – Cystinuria – Nuclear Magnetic Resonance – Urolythiasis – Urinalysis

Introduction

Cystinuria is an inherited transport disorder characterised by increased urinary excretion of cystine and other dibasic amino acids (arginine, lysine and ornithine). It results from the malfunction of a specific membrane transport system located in the brush-border membrane of the renal proximal straight tubule and the small intestine (Segal and Thier, 1990; Byrd et al., 1991). These

transport systems (probably three different proteins with different specificity) are specific for cystine, lysine, arginine and ornithine. The occurrence of such multiple transport systems is also supported by Brodehl (1975) and Scriver (1985), who found diminished reabsorption of cystine and dibasic amino acids in new-born human infants, and that the reabsorption capacity matured at different rates for each of the substrates. The gene responsible for cystinuria type I has been identified (Calonge et al., 1995; Pras et al., 1994) and the gene of type III is now under investigation (Bisceglia et al., 1997) as a consequence of the wide interest about the pathology in recent years.

The sole clinical manifestation is the formation of urinary calculi, being cystine only slightly water soluble. Untreated, the disorder leads to chronic infection, obstruction and renal failure. The data reported in literature show a great variation of incidence of the disease, from 1:600 to 1:17286 (Byrd et al., 1991), probably depending on the method used for population screening. Cystinuria accounts for 1 to 2% of all urolithiasis and 6 to 8% of urolithiasis in children. Because of its lifelong duration and the likelihood of recurring stones in affected individuals, cystinuria produces considerable morbidity. Cystinuria in excess of 0.42 mmol/L can be detected with cyanide-nitroprusside test (CNT), which is still widely used in screening for classic cystinuria, however, the reliability for detecting type II or type III heterozigotes is questionable. Semiquantitative thin layer chromatography (TLC) readily reveals cystinuria and iperdibasic aminoaciduria in homozigotes and compound heterozigotes. We present a Nuclear Magnetic Resonance (NMR) Spectroscopy procedure allowing, in few minutes, a more safe diagnosis when compared to the CNT test, while providing amino acid quantitation comparable with that obtained by other well established techniques such as amino acid analyzers of HPLC's (Livesey et al., 1996). Proton NMR Spectroscopy of urine (as well as of other biological fluids) is a very powerful technique enabling multi-component analyses that are already used for both diagnosis and follow-up of a wide range of inherited metabolic diseases (Pontoni et al., 1994).

The relevant amino acids can be detected in the urine NMR spectra of cystinuric patients (Fig. 1), the most abundant being lysine (5 mM and over are often detected), whose typical signals become very high; cystine, arginine and ornithine are also usually detectable, although pathologic concentrations are lower (usually below 2 mM).

Patients, materials and methods

Patients

Ten diagnosed patients, five male and five female subjects, whose age ranged from 9 to 19 years at the beginning were diagnosed and monitored during a two years period and urine levels of cystine, lysine and methionine were detected by NMR spectroscopy. The patients were treated with MPG (20 to 30 mg/Kg/die, adjusted according to the NMR test results), Captopril, 25 mg/die and, to alkalinise urine, sodium bicarbonate or, if NMR spectra indicated low levels of citric acid, sodium citrate was administered as needed.

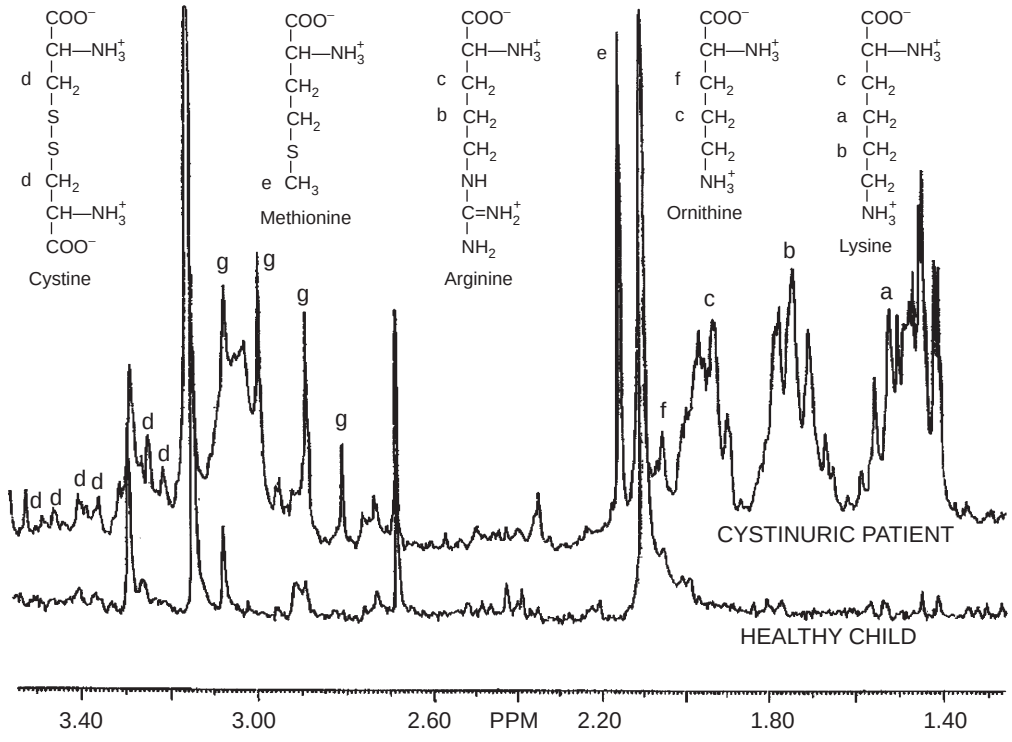

Fig. 1. NMR spectrum (1.2 to 3.4 ppm) of urine of a cystinuric patient (upper line) compared to that of a healthy child. The presence of the three high multiplets at 1.4 to 2 ppm (*a*, *b* and *c*) is indicative of the presence of lysine. Lower amounts of arginine and ornithine also give signals in the same spectral area. Six small signals of cystine are evidenced between 3.15 and 3.5 ppm (*d*) while the singlet at 2.13 (*e*) is assigned to methionine. In the spectrum the singlet (*f*) of creatinine and four peaks (*g*) of citric acid are also evidenced

NMR analysis

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. Acid environment is required in order to keep pH far from pKa of most organic acids, in order to minimise 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 analysed in a Bruker AC-200 E, a 4.7 Tesla Nuclear Magnetic Resonance Spectrometer 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. 1 were assigned by comparison with standard solutions and by addition in urine specimens and used for quantitation of the metabolites. Area estimates of the peaks are performed by the computer of the spectrometer. Lysine, cystine and methionine can be directly evaluated by means of specific peaks (a, d and e, respectively, in Fig. 1), usually without significant interference by other urine components, particularly when the aminoacids are accumulating in big excess as in cystinuria, quantitation of arginine and ornithine is somewhat more cumbersome, (based on area of common peaks b, c and f, Fig. 1). For routine analyses, 128 scans of 3.15 sec were accumulated. The water peak was eliminated by off resonance gate decoupling. All other

NMR experimental details were according to Lehnert and Hunkler (1986) with minor modifications.

HPLC analysis

A number of such urine specimen (8 cystinuric patients and 1 control) underwent aminoacid analysis by both HPLC and NMR methods. The HPLC analyses were performed on a fully equipped Beckman Gold chromatograph, equipped with a diode array UV-visible detector, an integrator, a post-column ninhydrin reactor, a programmable column temperature control unit and a Beckman Spherogel polystyrene-divynilbenzene sulfonate column for amino acids, using as the eluent a binary gradient of commercially available Beckman buffers (pH ranging from 2.75 to 3.8), following a Beckman standard protocol. Each analysis required 115 min of chromatographic run plus 30 min for regeneration.

Results

HPLC versus NMR comparison

The proposed NMR method was initially validated by performing calibration lines correlating NMR integrals with known concentrations of amino acids in plain water at pH 2.5. In Fig. 2, the data related to the four dibasic amino acids, hereby of relevance, are reported; as it can be noted, excellent correlations (R's) were obtained under these conditions.

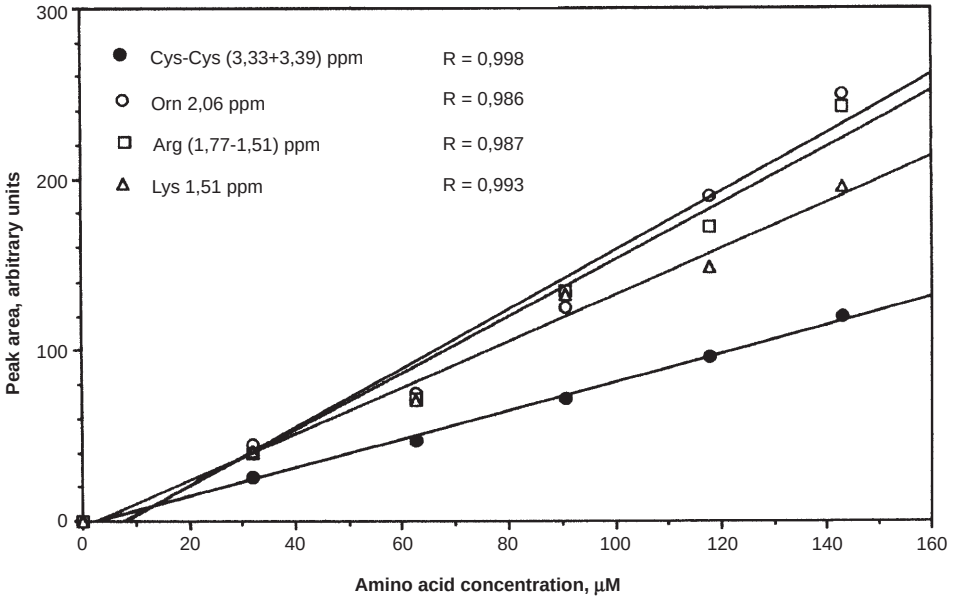

Fig. 2. Calibration plot of selected NMR peak integrals of dibasic aminoacids versus the known concentration of the same amino acids. The peaks centred at the reported ppm have been selected for quantitation (see Fig. 1). See also under "Patients, material and methods" for further details

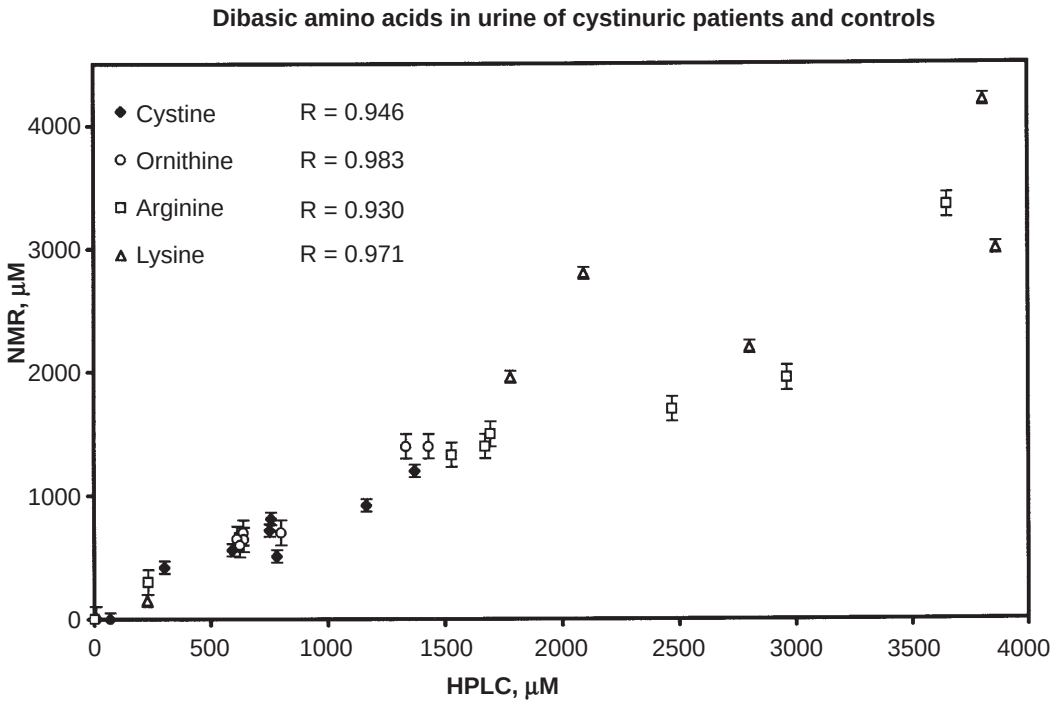

Fig. 3. Correlation plots of NMR-measured levels of cystine, lysine (two experimental points are out of scale), arginine and ornithine versus the corresponding HPLC estimated levels

In order to definitely validate the proposed NMR technique for measuring amino acid urine levels, the concentrations of cystine, lysine, ornithine and arginine have been plotted versus the corresponding levels as estimated by means of HPLC, probably today the most widely accepted analytical method for amino acid analysis together with the amino acid analyser.

Correlation of NMR results to HPLC data are shown in Fig. 3. From the calculated values of R's values ranging from 0.941 to 0.993 a satisfactory correspondence of the two techniques in measuring amino acid levels can be clearly evinced; in terms of diagnostic usefulness, no ambiguous results of the NMR method are to be expected.

To confirm the validity of the method, the age-related normal ranges of several amino acids have been calculated using at least 15 urine specimens of normal paediatric subjects. The NMR normal ranges are perfectly overlapping the reported normal ranges calculated by amino acid analyser (Pontoni et al., 1996).

Diagnosis

In Fig. 1 a typical spectrum of urine of a cystinuric patient in which the peaks assigned to the cystine and lysine are evidenced. The shape of the illustrated spectrum is by itself a sort of fingerprint for cystinuria. As it can be noted, the

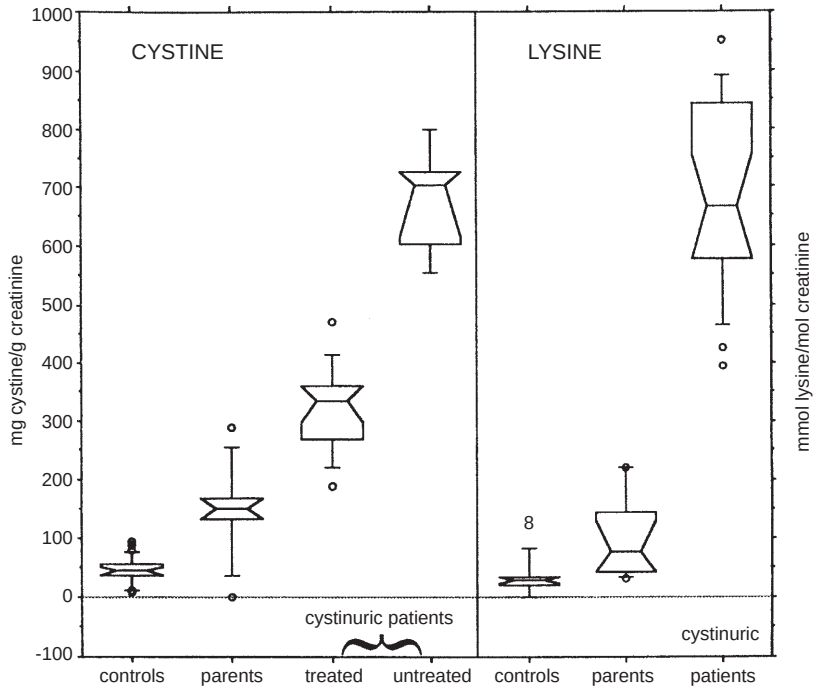

Fig. 4. Box and whiskers plot of cystine and lysine in the urine of groups of subjects. Normal values of both cystine and lysine are calculated by NMR on over 100 normal subjects. The parents of cystinuric patients (14 subjects) present levels of cystine and lysine statistically higher than normal subjects, but lower than cystinuric patients, as it may be expected for heterozigotes. The detected amounts of cystine in cystinuric patients are treated as two distinct set of data depending on whether the determination was performed under therapy (26 points) or not (12 points)

peak of cystine in cystinuria is lower than that of lysine. Moreover, in the spectral region where cystine resonates, high interference makes the cystine barely detectable when in physiological amounts. While lysine is more helpful in diagnosis, owing to its higher signals, the quantitation of cystine becomes more important during the follow-up of treatment, being the target of the therapy. Creatinine, citrate and methionine are also clearly detectable in Fig. 1; Creatinine is used to refer the amino acid content per gram of creatinine; citrate is sometimes administered during the therapy (to increase the urine pH where cystine is more soluble, Dent and Senior, 1955) and methionine is found increased in several patients.

Lysine and cystine contents in urine have been statistically evaluated as diagnosis indicators in Fig. 4. The box and whiskers plots of urinary lysine and cystine levels in cystinuric patients show sets statistically well distinct from the controls, indicating how both the aminoacids are suitable for diagnosis.

Conversely, cystine and lysine urine levels of parents of cystinuric patients are well separated from their children's levels and form a different statistical set with respect to controls. Quantitation of these two amino acids can hence be used as markers for identification of heterozigotes.

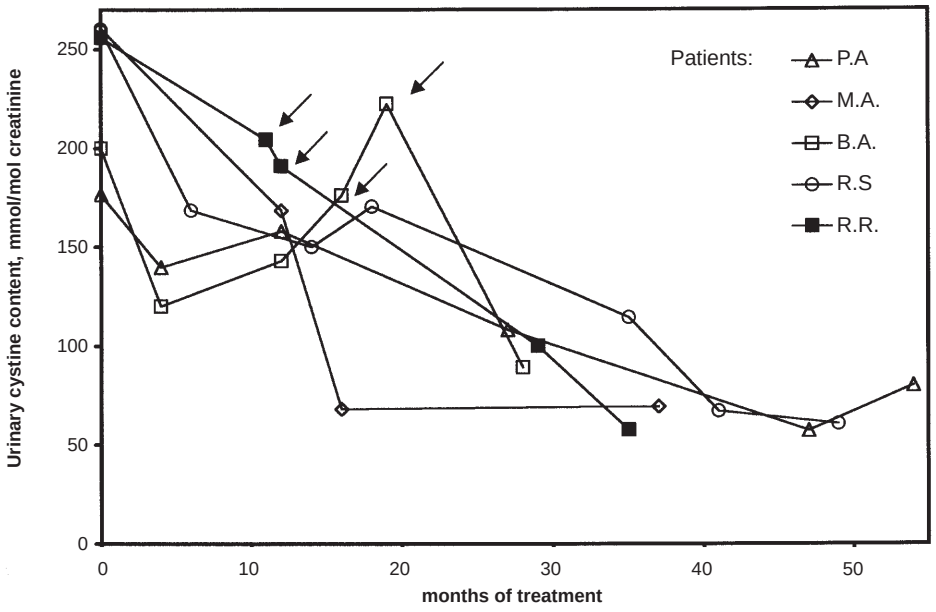

Fig. 5. NMR monitoring of cystine in cystinuric patients. All analyses were carried out under the therapy described in "Patients and methods" except at zero time (no therapy at all) and those pointed by arrows (no pharmacological therapy). Patients under therapy always show lower levels than untreated patients

Methionine, an aminoacid metabolically related to cystine, was found to be dramatically increased in five patients out of ten, whereas in the remaining five its levels were normal.

Therapy

In Fig. 5 the NMR monitoring of cystine excretion is illustrated in treated patients over a several years long time (only the points indicated by arrows and at zero time represent tests run on untreated patients) evidencing the efficacy of MPG therapy in lowering the cystine level below the 200 mmol/mol of Creatinine threshold. The lysine and methionine levels, instead, remain unchanged (data not shown).

Discussion

NMR spectra of urine, providing amino acid quantitation comparable with that obtained by other well established techniques such as HPLC allow, in few minutes, an unambiguous diagnosis and can be introduced in the clinical practice in confirming the Brandt test (often questionable). The proposed NMR technique is also suitable in the follow-up of therapy with MPG, because it simultaneously provides quantitation of cystine, citrates (also used during therapy to enhance urine pH and hence cystine solubility), and

creatinine, thus allowing the therapist to monitor cystine excretion in order to keep its level below the 200 mmol/mol of creatinine threshold, in order to optimise its solubilisation. It is also worth noting that in urine samples of heterozigotes, enhanced levels of lysine (though always below 1 mM, thus in concentrations unambiguously distinct from the urinary levels in homozigotes) can be detected, providing a very easy preliminary identification of heterozigotes.

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