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Short Communication

^{1}H NMR spectroscopy of body fluids in patients with inborn errors of purine and pyrimidine metabolism

R. A. WEVERS[1]*, U. ENGELKE[1], J. J. ROTTEVEEL[2], A. HEERSCHAP[3], J. G. N. DE JONG[1], N. G. G. M. ABELING[4], A. H. VAN GENNIP[4] and R. A. DE ABREU[2]

Institutes of [1]Neurology, [2]Paediatrics, [3]Radiology, University Hospital Nijmegen; [4]Laboratory of Procreation, University Hospital AMC Amsterdam, The Netherlands

**Correspondence: Institute of Neurology, University Hospital Nijmegen, Reinier Postlaan 4, 6500 HB Nijmegen, The Netherlands*

^{1}H NMR spectroscopy of body fluids has been used in the diagnosis of many inborn errors of metabolism (Lehnert and Hunkler 1986; Iles and Chalmers 1988). To our knowledge there is no systematic study available of body fluids from patients with inborn errors in the purine or pyrimidine metabolism. The main advantage of the technique over others that are used diagnostically in the screening for inborn errors of metabolism is the minimal sample pretreatment required for NMR spectroscopy. Fractionation, extraction or derivatization of metabolites is not required. A further advantage is the overall view of proton-containing metabolites. Quantification of metabolites is possible. An obvious disadvantage is the substantial cost of high-field NMR spectrometers that are required for this work. This paper demonstrates that NMR spectroscopy can be used in diagnosing inborn errors in purine and pyrimidine metabolism. Examples are given of NMR spectra of body fluids from patients with dihydropyrimidine dehydrogenase deficiency (McKusick 274270) and with dihydropyrimidinase deficiency (McKusick 222748).

METHODS

^{1}H NMR spectroscopy was carried out on 500 and 600MHz Bruker spectrometers at 298 K using 60° radiofrequency pulses and 6s pulse repetition time with 132 averages (Abeling et al 1995; Wevers et al 1994, 1995). Sample preparation included deproteinization of CSF (0.5ml) over a 10kDa filter (Sartorius Centrisart I, no. 13239, Göttingen, Germany) according to the instructions of the manufacturer. Subsequently 20 µl $^{2}H_2O$ containing trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (sodium salt; TSP) as internal standard was added to give a final concentration of 0.78mmol/L. The pH was adjusted to 2.50 ± 0.10 using a minimal volume of HCl. Urine was pretreated similarly but without deproteinization and by adding 50 µl instead of 20 µl $^{2}H_2O$ (final TSP concentration 1.84mmol/L).

GC-MS was carried out after ethyl acetate extraction of an acidified urine sample and trimethylsilylation. A capillary column (25m×0.22mm; fused-silica WCOT CP-Sil8CB (0.12μm)) was used for separation of pyrimidine bases and dihydropyrimidines. Electron-impact mass spectra were recorded on a VG Trio-2. Quantitative data on uracil and thymine were obtained from the GC analysis.

HPLC measurements were carried out in a laboratory that uses the technique in the screening for inborn errors of metabolism. Purine and pyrimidine nucleotides and bases were separated on a reversed-phase Supelcosil C18 10μm column and detected with a diode-array detector. Absorbance at 260nm was used to quantify the results.

RESULTS

Quantification of metabolites

To test the quantitative performance of GC-MS, HPLC and NMR spectroscopy in the analysis of pyrimidine bases, we added 0, 125 and 1250μmol/L of both uracil and thymine to a urine sample. Analysing laboratory technicians and staff were unaware of the spiking strategy used. The thymine concentration of the urine without addition was below the detection limit for all three techniques. For uracil there was an obvious quantitative discrepancy between the techniques. With GC it was found to be below the detection limit, but HPLC and NMR spectroscopy gave values of 335 and 57μmol/L, respectively. In the two samples that were spiked (lowest and highest concentration, respectively) the recovery with GC was 64% and 33% for uracil and 64% and 68% for thymine. With HPLC, uracil recovery amounted to 113% and 101%, while thymine was recovered at 174% and 99%. Recovery with NMR spectroscopy was 92% and 96%, for uracil and 103% and 105% for thymine.

Sensitivity and spectral characteristics of NMR spectroscopy

The sensitivity of NMR spectroscopy for each metabolite depends on the number of protons that contribute to a signal and the multiplicity of the resonance (Wevers et al 1994; 1995). In many cases the purines and pyrimidines have only one proton contributing to the signal. The sensitivity of the technique in such a case is 15μmol/L for a singlet resonance and 30μmol/L for a doublet resonance. Therefore, NMR spectroscopy does not seem sensitive enough for measurements in plasma or CSF of purine or pyrimidine metabolites when concentrations below 15μmol/L are to be expected. Some metabolites have no contributing proton in their chemical structure. For this reason metabolites like uric acid and 2,8-dihydroxyadenine are NMR invisible. Other molecules have more than one resonance in the spectrum. Such molecules can be recognized by their complete 'fingerprint' in the spectrum. Generally there is only minimal intersample variation in the chemical shift of a resonance (<0.01ppm), which allows reliable identification of the various purines and pyrimidines in urine, plasma and CSF. Hypoxanthine is an example of a metabolite with a more variable chemical shift; in particular, the 8.37ppm singlet resonance deriving from the C-2 proton has a higher intersample variation, which is pH dependent. At pH 2.50 its chemical shift ranges from 8.32 to 8.41ppm ($n=13$ urine samples). At pH 7.0 this resonance shifts to 8.21ppm. The second hypoxanthine resonance (C-8 proton, at pH 2.50

a singlet at 8.21ppm) showed less intersample variation in its chemical shift (range in 13 urine samples 8.202−8.224ppm). We therefore use the 8.21ppm singlet resonance for identification of hypoxanthine in the spectrum.

It is an advantage that many of the relevant compounds for the purine and pyrimidine pathways have a chemical shift between 5.5 and 9.0ppm, a region of the spectrum where few metabolites present in body fluids have resonances. Other metabolites known in this part of the spectrum are tyrosine, phenylalanine, formic acid, indoxyl sulphate, histidine and hippuric acid.

Examples of NMR spectra from patients with an enzyme defect

Dihydropyrimidine dehydrogenase (EC 1.3.1.2) deficiency: Figure 1 shows the urine NMR spectrum of a patient with this defect, showing a high uracil concentration (95 μmol/mmol creatinine) and also high thymine (270 μmol/mmol creatinine). In normal urine samples thymine cannot be detected with NMR spectroscopy, while uracil is observed in trace amounts in some samples (<10 μmol/mmol creatinine). 5-Hydroxymethyluracil, a metabolite of thymine, was not detectable in the sample.

Dihydropyrimidinase (EC 3.5.2.2) deficiency: We had the opportunity to measure urine, plasma and CSF of one patient with this disease. High concentrations in urine were found for uracil, thymine, dihydrouracil and dihydrothymine (144, 230, 490, 760 μmol/mmol creatinine, respectively). In plasma and CSF clearly increased levels were found for the dihydropyrimidines and slightly increased levels for uracil and thymine. Concentrations in CSF were higher than in plasma (for example, dihydrothymine 79 μmol/L in CSF versus 49 μmol/L in plasma; and dihydrouracil 46 μmol/L in CSF and below the detection limit (<15 μmol/L) in plasma). The absence of high concentrations of *N*-carbamyl-β-alanine (model compound Sigma no. C3750) excluded ureidopropionase deficiency as a possible defect in this patient. This finding suggested dihydropyrimidinase deficiency, which could later be confirmed enzymatically in the liver of the patient. In CSF, but not in urine or plasma of the patient, we found an as yet unidentifiable metabolite (triplet 2.61ppm, *J*-coupling 6.6Hz) that was never found in any of 64 other CSF samples of children suspected to have an inborn error of metabolism. Also, we never found this resonance in plasma or urine samples of other patients with unrelated diseases. Measurement of samples from other patients will show whether this metabolite occurs generally in this disease.

DISCUSSION

Quantitative comparison of GC, NMR and HPLC data for uracil and thymine showed unacceptable differences between the techniques. Especially in an unspiked urine sample, obviously higher values for uracil were obtained with HPLC than with NMR or GC-MS, suggesting that compounds other than uracil may have contributed to the HPLC result. Dietary substances with similar chromatographic behaviour (retention time, absorbance curves) may have influenced the HPLC results at 260nm. Full recovery of added thymine and uracil in two spiked urine samples was found using NMR spectroscopy, indicating that metabolites from purine and pyrimidine metabolism can be quantified reliably with NMR

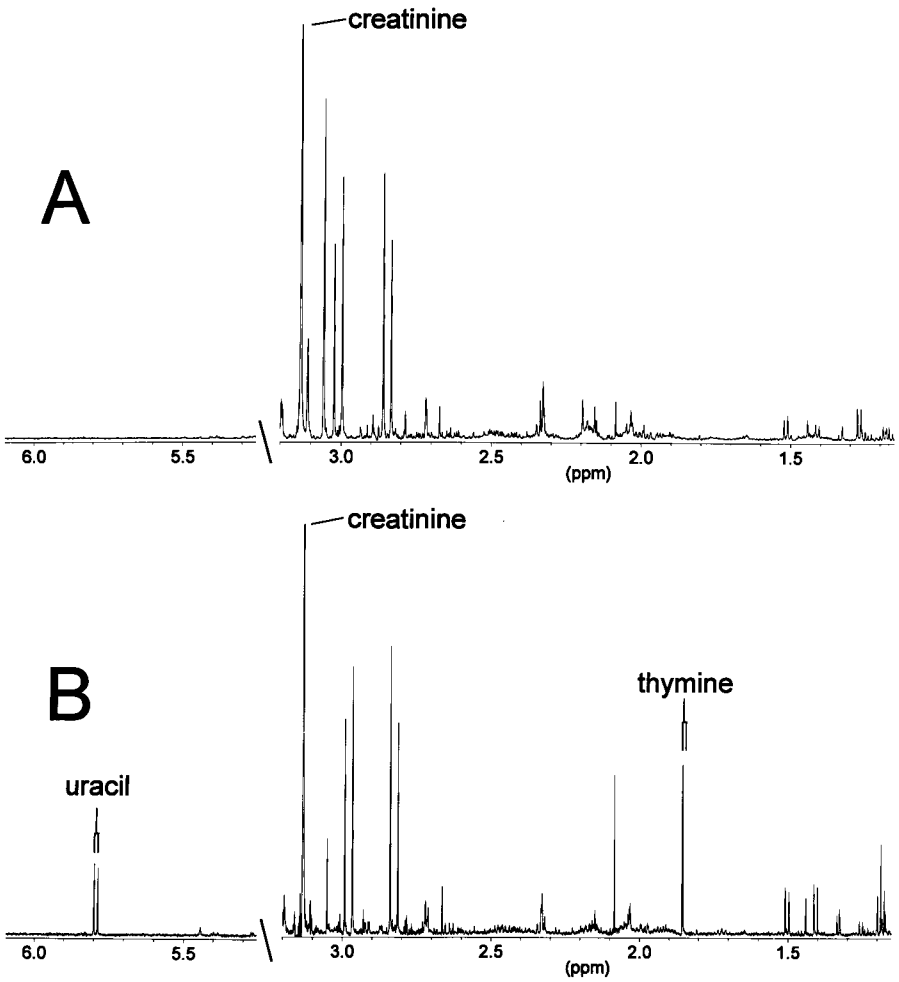

Figure 1 NMR spectra of urine samples from (A) a healthy individual, (B) a patient with dihydropyrimidine dehydrogenase deficiency, (C) a patient with dihydropyrimidinase deficiency. (D) Relevant metabolic pathway

spectroscopy. Using HPLC, thymine recovery was too high in one of the samples, while full uracil recovery was obtained in both samples. These findings suggest that quantification of purines and pyrimidines in urines is not unequivocal and call for incorporation of relevant purine and pyrimidine metabolites in quality control schemes used by diagnostic laboratories.

NMR spectroscopy measurements in urine samples were informative in the inborn errors shown in this paper. In dihydropyrimidine dehydrogenase deficiency and in dihydropyrimidinase deficiency, the NMR spectra of the urine samples led straightforwardly to the diagnosis. The absence of *N*-carbamyl-β-alanine in the urine spectrum of

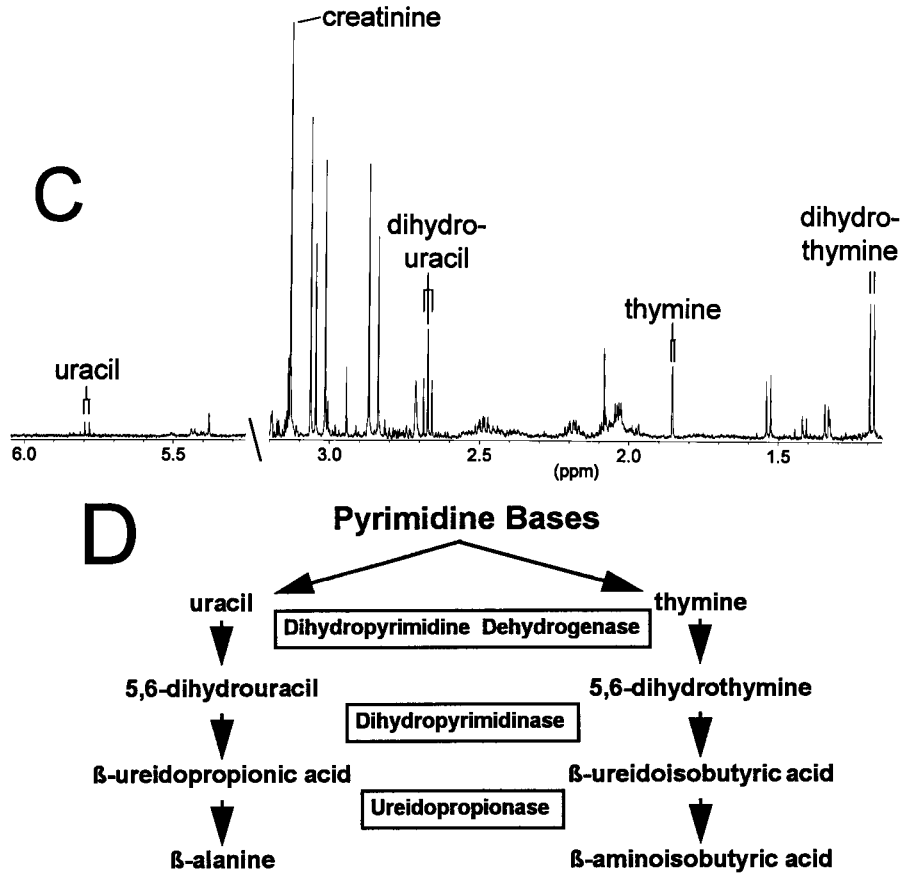

Figure 1 continued

the patient with dihydropyrimidinase deficiency excluded the theoretical possibility of ureidopropionase deficiency, a defect that has not yet been found in man. In this case NMR spectroscopy helped to pinpoint the enzyme defect. In dihydropyrimidinase deficiency as yet unknown resonances were demonstrated in the CSF of the patient. Such unknowns may be relevant for our understanding of the disease characteristics. It therefore seems important to study the occurrence of such metabolites in larger series of patients. It seems worthwhile also to study other inborn errors in purine and pyrimidine metabolism with NMR spectroscopy. As these diseases are very rare, the authors would appreciate if urine, plasma or CSF samples from well-documented cases were made available for NMR spectroscopy.

NMR spectroscopy provides an overall view on hydrogen-containing metabolites. This means that the technique may help in diagnosing patients with as yet unknown inborn errors in purine or pyrimidine metabolism. Assuming that an abnormal metabolite in such a patient has resonating protons and that its concentration in the body fluid is above the detection limit of the technique, one or more unusual resonances would be present in the

NMR spectrum. It then depends on the quality of the model compound NMR database available whether such resonances could be properly assigned. This is unlike the situation with conventional techniques, where special and often elaborate sample preparation in combination with thin-layer chromatography or HPLC is required to detect and to identify such a compound (van Gennip et al 1991, 1993). To make use of this characteristic of NMR spectroscopy it is required to measure NMR spectra of model compounds of as many intermediates from this part of metabolism as possible and to incorporate them in the model compound database. This is a topic of further research in this field.

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