N-Acetylated Metabolites in Urine: Proton Nuclear Magnetic Resonance Spectroscopic Study on Patients with Inborn Errors of Metabolism

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Background: There is no comprehensive analytical technique to analyze N-acetylated metabolites in urine. Many of these compounds are involved in inborn errors of metabolism. In the present study, we examined the potential of proton nuclear magnetic resonance (1H-NMR) spectroscopy as a tool to identify and quantify N-acetylated metabolites in urine of patients with various inborn errors of metabolism.

Methods: We performed 1H-NMR spectroscopy on a 500 MHz spectrometer. Using a combination of one- and two-dimensional correlation spectroscopy (COSY) 1H-NMR spectra, we were able to assign and quantify resonances of characteristic N-acetylated compounds produced in urine of patients with 13 inborn errors of metabolism.

Results: The disease-specific N-acetylated metabolites were excreted at concentrations >100 μmol/mmol of creatinine in the patients' urine. In control urine samples, the concentration of individual N-acetyl-containing compounds was <40 μmol/mmol of creatinine. The combination of one- and two-dimensional COSY NMR spectroscopy led to the correct diagnosis of nine different inborn errors of metabolism. No abnormalities were observed in the spectra of urine from patients with GM1- or GM2-gangliosidosis. We also determined the 1H-NMR characteristics of N-acetylated metabolites that may be relevant to human metabolism.

Conclusion: 1H-NMR spectroscopy may be used to identify and quantify N-acetylated metabolites of diagnostic importance for the field of inborn errors of metabolism.

Proton nuclear magnetic resonance (1H-NMR) spectroscopy of body fluids has been used to diagnose inborn errors of metabolism (1–3). The technique yields an overview of proton-containing metabolites, is rapid and nondestructive, and requires minimal sample pretreatment. In this report, we document the effective use of 1H-NMR spectroscopy for the identification and quantification of N-acetyl-containing metabolites in body fluids of patients with various inborn errors of metabolism. N-Acetylation occurs in many metabolic pathways, which makes the detection of N-acetylated compounds important. Several diseases are known to be associated with the accumulation of N-acetylated metabolites. Some of these have been studied by 1H-NMR spectroscopy. The urinary excretion of N-acetylated amino acids has been published for patients with inborn errors of amino acid metabolism (4, 5). N-Acetylaspartic acid and N-acetylaspartylglutamic acid are the laboratory marker metabolites in Canavan disease (6). Other authors have described one- and two-dimensional NMR experiments for structural characterization and identification of N-acetylneuraminic acid-containing storage products in lysosomal diseases (7–9). These studies demonstrate that the detection of N-acetylated metabolites assists in the diagnosis of various inborn errors of metabolism. However, many N-acetylated compounds remain undetected by the conventional analytical techniques used in metabolic screening laboratories.

The three equivalent N-acetyl protons resonate as a singlet in a small part of the 1H-NMR spectrum (1.9–2.2

Nonstandard abbreviations: 1H-NMR, proton nuclear magnetic resonance; TLC, thin-layer chromatography; GlcNAc, N-acetylgalactosamine; COSY, correlation spectroscopy; and ISSD, infantile sialic acid storage disorder.

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ppm). Only a few other metabolites are known to cause resonances in this area. The finding of singlet signals in a one-dimensional proton NMR spectrum can thus be used as an indication of the presence of an N-acetylated metabolite.

We present here the NMR characteristics of many N-acetylated compounds relevant for human metabolism. In addition, we provide NMR spectra for urines representative for 13 inborn errors of metabolism that involve N-acetylated compounds.

Materials and Methods

Model Compounds

Most model compounds were commercially available from Sigma or Aldrich. N-Acetylated oligosaccharides from lysosomal storage diseases were isolated preparatively from thin-layer chromatography (TLC) plates routinely used for mono- and oligosaccharide analysis from urine of affected patients. Manα1-3Manβ1-4-N-acetylgalacosamine (GlcNAc), Fuc-GlcNAc-Asn, aspartylglucosamine, and two sialyloligosaccharides were isolated with a butanol–acetic acid–water (2:1:1 by volume) solvent system and visualized with orcinol-sulfuric acid (Fig. 1). Because of an overlap with lactose, Manβ1-4GlcNAc from the urine of a β-mannosidosis patient was isolated with propanol–acetic acid–water (42.5:0.5:7.5 by volume) solvent system. The characteristic TLC bands were removed preparatively from the silica-coated plate and measured as a model compound.

Urine

Control urine samples were from 10 healthy individuals and from 50 diseased patients. In the urine samples from diseased controls sent to our laboratory, extensive metabolic screening had ruled out any relevant inborn errors of metabolism.

Urine was centrifuged before analysis. We added 70 μL of a 20.2 mmol/L trimethylsilyl-2,2,3,3-tetradeteropropionic acid (sodium salt; Aldrich) 2H2O solution to 700 μL of urine as a chemical shift reference (δ = 0.00) and as a lock signal. The pH of the urine was adjusted to 2.50 ± 0.05 with concentrated HCl. Finally, we placed 650 μL of the sample in a 5-mm NMR tube (Wilmad Royal Imperial).

NMR Measurements

High-resolution spectra for urine samples and the model compounds were acquired at 500 MHz on a Bruker DRX and AMX spectrometer equipped with a sample changer. The spin-lattice or longitudinal relaxation time (T1) values for N-acetylmuramic acid, N-asparaglycosamine, N-acetylaspartic acid, and Man(1-4)GlcNAc, as representative N-acetylated compounds, were determined in urine of patients by the inversion-recovery technique. The T1 values amounted to 1.0, 0.9, 1.6, and 0.9 s, respectively.

One-dimensional 1H-NMR Spectroscopy

For urine, 128 transients were collected into 32,000 data points with a spectral width of 6002 Hz. The H2O resonance was presaturated during the relaxation delay (10 s). Shimming of the sample was performed automatically on the deuterium signal.

A sine-bell squared filter (SSB = 2) was used, and spectra were Fourier-transformed after the free induction decay was zero-filled to 64,000 data points. The phase and the baseline were corrected manually. The N-acetyl resonances in the one-dimensional urine spectra were fitted semi-automatically to a Lorentzian line shape model function. The integrals of these fits were used for quantification by comparing them to the fit integral of creatinine (singlet at 3.13 ppm). For identification and quantification of the NMR spectra, 1D WinNM and the AMIX software were used (Bruker BioSpin). A prerequisite for accurate quantitative determination of metabolites with proton NMR spectroscopy lies in the length of the relaxation delay. We used a delay of 10 s, which is more than five times the T1 value of the N-acetyl protons in N-acetylaspartic acid, the compound with the longest T1 value among the N-acetylated compounds.

1H–1H Correlation Spectroscopy (COSY)

The spectral widths in the F1 and F2 axes were 6002 Hz, and 4000 data points were collected in F2. For the urine samples, 256 increments and 16 scans per increment were used. The relaxation delay was 2 s, and before the Fourier transformation, a sine function was applied in both time

Fig. 1. Thin-layer chromatogram of dextran and urine samples.

The solvent system was butanol–acetic acid–water or orcinol–sulfuric acid. Lanes: 1, dextran; 2, sample from a patient with fucosidosis; 3, sample from a patient with α-mannosidosis; 4, sample from a patient with α-galactosidosis; 5, sample from a patient with α-1,6-galactosidosis; 6, dextran; 7, sample from a healthy individual; 8, sample from a patient with aspartylglucosaminuria; 9, sample from a patient with sialidosis. The arrows indicate ???.

Engelke et al.: 1H-NMR Spectroscopy of N-Acetylated Metabolites
domains. During the relaxation delay, the water resonance was presaturated.

QUANTITATIVE DATA

To study the linearity and recovery, we added three N-acetylated metabolites, N-acetytyrosine (cat. no. A-2513; Sigma), N-acetyltryptophan (cat. no. A-6376; Sigma), and N-acetylaspartic acid (cat. no. A-5625; Sigma), separately to normal human urine in concentrations of 0, 100, 500, and 3000 μmol/L. After addition of these metabolites, the creatinine concentration of the urine was 1.1 mmol/L. The same experiment was repeated with a second urine with a creatinine concentration of 2.0 mmol/L. For concentration calculations for the N-acetylated metabolites using 1H-NMR spectroscopy, we used trimethylsilyl-2,2,3,3-tetradeteropropionic acid as the concentration reference, and the metabolite concentrations were expressed in μmol/L.

Results

N-ACETYL RESONANCES IN URINE

A representative 1H-NMR spectrum of urine from a healthy individual is shown in Fig. 2. Methyl protons from N-acetyl groups characteristically give a singlet resonance in a limited part of the 1H-NMR spectrum (1.9–2.2 ppm). Table 1 provides an overview of the N-acetyl-containing metabolites detectable in urine samples from healthy individuals and patients. Individual compounds can be recognized by the chemical shift of the N-acetyl group CH3 protons and from resonances deriving from other parts of the molecule. Neuraminic acid, N-acetylaspartic acid, and the N-acetyl groups in glycopeptides in urine from healthy controls are known to

Table 1. 1H resonance positions and multiplicity of N-acetyl-containing metabolites.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>N-CH3 singlet(s)</th>
<th>Rest of the molecule*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Neuraminic acid (β anomer)</td>
<td>2.05</td>
<td>1.85 (dd) ↔ 2.26 (dd)</td>
</tr>
<tr>
<td>N-Neuraminic acid (α anomer)</td>
<td>2.03</td>
<td>1.65 (dd) ↔ 2.72 (dd)</td>
</tr>
<tr>
<td>N-Neuraminic acid (Bound AcNeu2—6)</td>
<td>2.02; 2.03; 2.06</td>
<td>1.72 (dd) ↔ 2.67 (dd)</td>
</tr>
<tr>
<td>GlcNAc-Asn</td>
<td>2.01</td>
<td>2.97 (mu) ↔ 4.11 (tr); 3.83 (mu) ↔ 5.08 (do); 3.87 (mu) ↔ 5.09 (do)</td>
</tr>
<tr>
<td>(GalGlcNAc)-Asn</td>
<td>2.00</td>
<td>2.95 (mu) ↔ 4.10 (tr); 3.54 (mu) ↔ 4.48 (do); 3.87 (mu) ↔ 5.09 (do)</td>
</tr>
<tr>
<td>Man(1-3)GlcNAc</td>
<td>2.03</td>
<td>3.72 (mu) ↔ 4.72 (do); 3.89 (mu) ↔ 5.20 (do)</td>
</tr>
<tr>
<td>N-Acetyltyrosine</td>
<td>2.03</td>
<td>1.52 (mu); 1.65–1.80 (mu); 3.12 (qu)</td>
</tr>
<tr>
<td>N-Acetylaspartic acid</td>
<td>2.03</td>
<td>2.95 (mu) ↔ 4.72 (mu)</td>
</tr>
<tr>
<td>N-Acetyltirosine</td>
<td>1.94</td>
<td>3.07 (AB) ↔ 4.57 (mu)</td>
</tr>
<tr>
<td>Man(1-3)Man(1-4)GlcNAc</td>
<td>2.03</td>
<td>3.88 (mu) ↔ 5.20 (do); 4.04 (do) ↔ 5.10 (mu)</td>
</tr>
<tr>
<td>Fuc-GlcNAc-Asn</td>
<td>2.01</td>
<td>1.21 (do) ↔ 4.12 (qu); 2.94 (mu) ↔ 4.06 (tr); 3.78 (do) ↔ 4.90 (do); 3.83 (mu) ↔ 5.10 (do)</td>
</tr>
<tr>
<td>β-GlcNAc(1-2)αMan(1-3) \ βMan(1-4)GlcNAc</td>
<td>2.05; 205</td>
<td>4.10 (mu); 4.19 (mu); 4.24 (mu); 4.53 (do); 5.11 (mu); 5.20 (do)</td>
</tr>
<tr>
<td>β-GlcNAc(1-2)αMan(1-3) / β-GlcNAc(1-4)αMan(1-3)</td>
<td>2.04; 2.05; 2.06</td>
<td>4.10 (mu); 4.19 (mu); 4.24 (mu); 4.45 (do); 4.53 (do); 5.05 (mu); 5.11 (mu); 5.20 (do)</td>
</tr>
<tr>
<td>β-GlcNAc(1-2)αMan(1-3) \ β-GlcNAc(1-4)αMan(1-3)</td>
<td>2.04</td>
<td>1.21 (do); 1.23 (do); 4.42 (mu); 5.13 (do); 5.15 (do); 5.18 (do); 5.29 (do); 5.38 (do); 5.45 (si)</td>
</tr>
<tr>
<td>GalNAcFuc (Blood group A trisaccharide)</td>
<td>2.04</td>
<td>3.19 (si)</td>
</tr>
<tr>
<td>Acetylcarnitine</td>
<td>2.14</td>
<td>3.19 (si)</td>
</tr>
<tr>
<td>N-Acetylglutamic acid</td>
<td>2.03</td>
<td>2.00 (mu); 4.38 (mu); 2.49 (tr); 2.19 (mu)</td>
</tr>
<tr>
<td>Biantennary octasaccharide (in Gm1,gangliosidosis urine)</td>
<td>2.04; 2.05; 2.05</td>
<td>4.11 (do); 4.19 (do); 4.25 (do); 4.46 (do); 4.57 (do); 5.11 (mu); 5.20 (do)</td>
</tr>
<tr>
<td>N-Acetylaspartylglutamic acid</td>
<td>2.04</td>
<td>2.01 (mu); 2.23 (mu); 2.48 (mu); 4.44 (mu); 2.85 (AB); 4.72 (mu)</td>
</tr>
<tr>
<td>N-Acetyltirosine</td>
<td>1.92</td>
<td>3.31 (mu) ↔ 4.70 (qu); 7.17 (tr); 7.25 (tr); 7.26 (si); 7.50 (do); 7.67 (do)</td>
</tr>
</tbody>
</table>

* ↔, cross-peaks observed in two-dimensional COSY NMR spectra of urine from patients who were investigated; si, singlet; do, doublet; tr, triplet; qu, quartet; mu, multiplet; dd, doublet-doublet; AB, AB-system.

b Data from the literature (22).
contribute to the resonances in this area (10). In addition, a few metabolites without an N-acetyl group are known to resonate between 1.9 and 2.2 ppm. These include glutamine (β-CH₂ multiplet at 2.15 ppm), proline (β-CH₂ multiplet at 2.02 ppm), methionine (S-CH₃ singlet at 2.13 ppm) (11) and acetic acid (CH₃ singlet at 2.08 ppm).

**QUANTITATIVE DATA**
The linearity was excellent for all three N-acetylated metabolites, N-acetyltirosine, N-acetyltryptophan, and N-acetylaspartic acid, (slope, 0.94–1.09; intercept did not significantly deviate from zero; correlation coefficient >0.99). The recovery was in 90–110% for these compounds at all concentrations.

To study the reproducibility (within-run), we prepared and measured 10 samples from the same urine on the same day. The within-run CV was <4% for all three N-acetylated metabolites.

**STRATEGY FOR QUANTITATIVE INTERPRETATION AND ASSIGNMENT**
Because of the low concentrations of N-acetylated metabolites, assignment of their singlet resonances in urine from healthy volunteers is generally not possible. Metabolic diseases that involve N-acetylated compounds may be diagnosed if the concentrations of the characteristic N-acetylated metabolite are high enough to qualify them as abnormal and to allow their identification. Identification requires the spectral information from the protons in the rest of the molecule (Table 1). As a systematic approach, we quantified the highest singlet resonance between 1.9 and 2.2 ppm. Excluded from this quantification was the singlet for acetic acid (mean chemical shift, 2.083 ppm; minimum, 2.081 ppm; maximum, 2.085 ppm; n = 10). It was assumed that CH₃ protons from N-acetylated metabolites cause the resonances in this region. Fig. 3 shows the concentrations of individual N-acetyl-containing compounds in urine samples from 50 diseased controls and 10 healthy volunteers. The highest concentration of an N-acetylated compound was generally <40 μmol/mmol of creatinine. Higher concentrations were observed in children under 5 years. Especially in the first month of life, values up to 200 μmol/mmol of creatinine could be found. Fig. 3 also shows data on urine samples from patients with 13 different inborn errors of metabolism involving N-acetylated compounds <40 μmol/mmol of creatinine. The CH₃ protons from the N-acetyl group are the only resonances from most N-acetylated compounds that can be observed in the spectra. Therefore, assignments based solely on the N-acetyl singlet were not possible. As shown in Fig. 3, most patients with inborn errors of metabolism excreted the N-acetylated metabolites typical for their diseases at concentrations >100 μmol/mmol of creatinine. At such concentrations, the typical N-acetyl compound could be assigned with greater certainty because the other protons of the molecule contribute to the assignment. Two-dimensional COSY spectra may help with further identification of relevant metabolites. As an example, Fig. 4 shows how specific cross-peaks in a two-dimensional COSY spectrum of the model compound Fuc-GlcNAc-Asn (Fig. 4A) can help to identify this metabolite in the urine of a patient with fucosidosis (Fig. 4B). The cross-peaks derive from the GlcNAc protons (3.83–5.10 and 3.78–4.90 ppm), the Asn protons (2.94–4.06 ppm), and the Fuc protons (1.21–4.12 ppm).

Fig. 3. Quantification of the N-acetylated compound with the highest concentration in the urine as function of age.

○, controls; ●, urine of affected patients. 1, Salla disease; 2, French-type sialuria; 3, α-mannosidosis; 4, β-mannosidosis; 5, fucosidosis; 6, aspartylglucosaminuria; 7, GM₁-gangliosidosis; 8, GM₂-gangliosidosis; 9, sialidosis; 10, Canavan disease; 11, citrullinemia; 12, tyrosinemia type I; 13, tyrosinemia type II.

Fig. 4. COSY spectra of model compound Fuc-GlcNAc-Asn obtained from TLC chromatography (A) and urine from a patient with fucosidosis (B). The characteristic pattern of the model compound Fuc-GlcNAc-Asn is shown in the circled cross-peaks.
INBORN ERRORS OF METABOLISM
Infantile sialic acid storage disorder (ISSD; OMIM 269920)/
Salla disease (OMIM 604369). ISSD and Salla disease are both characterized by increased concentrations of free sialic acid (N-acetylneuraminic acid) in the urine and by its storage in the lysosomes of different tissues. The molecular basis of these diseases is a defect in the lysosomal sialic acid transporter. Severe mutations in the gene lead to ISSD, whereas milder mutations clinically present as Salla disease. Characteristically, a 20- to 200-fold increase in N-acetylneuraminic acid excretion in urine has been described in infantile cases of the disease. Bound sialic acid typically is within the range obtained for controls.

One- and two-dimensional COSY $^1$H-NMR spectra were obtained from urine of a 16-year-old patient with Salla disease. The one-dimensional urine spectrum showed the presence of N-acetylneuraminic acid [singlet at 2.05 ppm; 121 µmol/mmol of creatinine; reference values, 20.2 (11.6) µmol/mmol of creatinine for ages >2 years (12); (Fig. 5A)]. The COSY spectrum confirmed the presence of free N-acetylneuraminic acid by showing

Fig. 5. 500 MHz $^1$H-NMR spectra of urine samples.
(A), Salla disease; (B), French-type sialuria; (C), α-mannosidosis; (D), β-mannosidosis; (E), fucosidosis; (F), aspartylglucosaminuria; (G), GM1-gangliosidosis; (H), GM2-gangliosidosis; (I), sialidosis; (J), Canavan disease; (K), citrullinemia; (L), tyrosinemia type I. The creatinine peak (singlet at 3.13 ppm; not shown) in the urine spectra is scaled to 100.
cross-peaks from the pyranose ring protons of the carbon-3 atom (1.85 and 2.26 ppm; data not shown) (13).

French-type sialuria (OMIM 269921). French-type sialuria is a metabolic disorder caused by a defect in the enzyme uridine diphosphate-N-acetylglucosamine 2-epimerase (EC 5.1.3.14), which is involved in N-acetylneuraminic acid synthesis. The defect in the enzyme leads to failing feedback inhibition by N-acetylneuraminic. Free N-acetylneuraminic acid is massively increased in the body fluids of diseased patients. We investigated the urine of a 6-year-old child with this disease. N-Acetylneuraminic acid excretion (Fig. 5B) was much higher than in the patient with Salla disease [4275 μmol/mmol of creatinine; reference values, 20.2 (11.6) μmol/mmol of creatinine for ages >2 years (12)].

Because of the very high concentration of N-acetylneuraminic acid, even the pyranose ring protons (3.5–4.1 ppm) and the nitrogen proton (doublet at 8.09 ppm) were clearly observed in the one- and two-dimensional spectra (data not shown).

α-Mannosidosis (OMIM 248500). Patients with a deficiency of the lysosomal enzyme α-mannosidase (EC 3.2.1.24) have high concentrations of Man₉GlcNAc (n ≥2) oligosaccharides in the cells and urine.

Oligosaccharide TLC of the urine from an affected patient showed many mannose-rich oligosaccharide bands. The major oligosaccharide band (Fig. 1, lane 2) typical for this defect was isolated preparatively from the silica-coated plate and measured as a model compound by ¹H-NMR. The compound was identified by comparison with reference spectra as the trisaccharide Man₃-1-3Manβ1-4GlcNAc (14).

One- and two-dimensional COSY ¹H-NMR spectra were obtained from urine of the patient. In the COSY spectrum, Manα1-3Manβ1-4GlcNAc was recognizable as two cross-peaks (4.04–5.10 and 3.88–5.20 ppm), and in the one-dimensional spectrum was recognizable as a singlet at 2.03 ppm (Fig. 5C) and doublet at 5.20 ppm. The urinary concentration of Manα1-3Manβ1-4GlcNAc in our patient was 153 μmol/mmol of creatinine.

β-Mannosidosis (OMIM 248510). The lysosomal enzyme β-mannosidase is involved in glycoprotein catabolism. A deficiency of this enzyme leads to excessive urinary excretion of the disaccharide Manβ1-4GlcNAc.

We measured urine samples from two brothers (ages 8 and 9 years) with β-mannosidosis. Oligosaccharide TLC was performed on urine samples from the boys. The Manβ1-4GlcNAc band (data not shown) was isolated preparatively from the silica-coated plate (100 μg) and measured as a model compound by ¹H-NMR. The spectrum showed the N-acetyl singlet (2.03 ppm), the 1α and 1β GlcNAc protons (doublets at 4.72 and 5.20 ppm, respectively), and many resonances in the 3.4–4.1 region deriving from the GlcNAc and Man parts of the disaccharide. The one-dimensional NMR spectrum of their urine showed the same singlets at 2.04 ppm (Fig. 5D) and doublets at 5.21 ppm. The doublet at 4.72 ppm was not visible because of overlap of the water resonance. The COSY spectrum from the urine showed characteristic cross-peaks from the 1α and 1β GlcNAc protons (3.89–5.20 and 3.72–4.72 ppm, respectively; data not shown). The concentration of Manβ1-4GlcNAc in the urine of the brothers was 255 and 273 μmol/mmol of creatinine.

Another, smaller singlet at 2.03 ppm was also observed in the one-dimensional NMR spectra of the two brothers (56 and 122 μmol/mmol of creatinine). Presumably this compound is sialyl-α2-6-mannosyl-β1-4-N-acetylglucosamine (~10% of the concentration of Manβ1-4GlcNAc (7)). Van Pelt et al. have identified this trisaccharide in concentrated urine fractions of affected patients.

Fucosidosis (OMIM 230000). Because of a deficiency of lysosomal α-L-fucosidase (EC 3.2.1.51), patients with fucosidosis accumulate fucose-containing glycolipids, oligosaccharides, or glycosphingolipids in the urine and tissue. Three characteristic oligosaccharides were isolated from urine of an affected patient by TLC (Fig. 1, lane 3). These bands were removed from the TLC plate and dissolved in ²H₂O. The fractions were analyzed by ¹H-NMR spectroscopy and compared with the literature (15). The one- and two-dimensional spectra of fraction 1 indicated the presence of Fuc-GlcNAc-Asn (Table 1). Because of the low concentrations in the isolated fractions 2 and 3, interpretation was impossible.

The characteristic COSY pattern of Fuc-GlcNAc-Asn (cross-peaks at 1.21–4.12 and 2.94–4.06 ppm) was observed in the spectrum for urine from an affected adult patient (Fig. 4B). The urinary Fuc-GlcNAc-Asn concentration was 109 μmol/mmol of creatinine (singlet at 2.01 ppm; Fig. 5E).

Aspartylglucosaminidase deficiency or aspartylglucosaminuria is an inborn error of metabolism caused by the deficiency of the lysosomal enzyme N-aspartyl-β-glucosaminidase (EC 3.5.1.26). This enzyme cleaves the N-acetylglucosamine-glycosamine linkage found in a variety of oligosaccharides and glycoproteins. Patients with the disease excrete aspartylglucosaminuria (GlcNAc-Asn) as major abnormal metabolite. In addition, several other glycosaminoglycans, such as Galβ1-4GlcNAcβ1-N-Asn, the mannose-rich derivative Man-Man-GlcNAc-GlcNAc-Asn, and NeuAc-Gal-GlcNAc-Asn, can be excreted (16). Urine samples from two unrelated cases were available. TLC with orcinol and ninhydrin showed the abnormal Galβ1-4GlcNAcβ1-N-Asn band in these samples (Fig. 1, lane 9). TLC plates stained with ninhydrin showed the GlcNAc-Asn and the Galβ1-4GlcNAcβ1-N-Asn band in the urine (data not shown). The NMR spectra of nearly pure compounds were compared with reference spectra from the literature (17). The resonances of both compounds are shown in Table 1. The
characteristic cross-peaks of both compounds were observed in COSY spectra of urine from both patients. The GlcNAc-Asn concentrations (singlet at 2.02 ppm; Fig. 5F) were 169 and 323 μmol/mmol of creatinine, respectively. The Galβ1-4GlcNAcβ1-N-Asn (singlet at 2.01 ppm) concentrations were 27 (case 1) and 114 μmol/mmol of creatinine (case 2).

G\textsubscript{M\textsubscript{2}}-Gangliosidosis (OMIM 230500). A deficiency of β-d-galactosidase (EC 3.2.1.23) leads to G\textsubscript{M\textsubscript{2}}-gangliosidosis. Oligosaccharides with a d-galactosyl group at the nonreducing end are increased in the urine of affected patients.

Three bands were isolated preparatively by TLC from the urine of an affected patient (Fig. 1, lane 5) and compared with spectra from the literature (17). The NMR spectrum of band 2 corresponded to the biantennary octasaccharide (17), and the NMR spectrum of band 3 corresponded to the triantennary decasaccharide (17) or the tetraantennary dodecasaccharide (17). For band 1, we could not find an exact match with reference spectra.

The one-dimensional NMR spectrum of urine from an affected patient (Fig. 5G) showed singlets between 2.00 and 2.04 ppm. The singlet with the highest intensity (2.03 ppm) represented a N-acetylated storage compound with a concentration of 34 μmol/mmol of creatinine.

\( G_{M2}-Gangliosidosis \) (Sandhoff disease; OMIM 268800). As a result of a deficiency in β-N-acetylgalactosaminidase (EC 3.2.1.53), oligosaccharides containing N-acetylgalactosamine are excreted in the urine of affected patients.

Using TLC, we were able to preparatively isolated two bands from the urine of an affected patient (Fig. 1, lane 4). The NMR spectra of both isolated bands showed resonances at 5.20, 5.11, and 4.53 and a singlet at 2.05 ppm. Band 2 differed from band 1 by an additional doublet at 4.46 ppm and two more singlets (2.045 and 2.06 ppm). The chemical shift data point to oligosaccharides with the structure β-GlcNAc-(1→2)-α-Man-(1-3)β-GlcNAc-(1→2)-α-Man-(1→4)β-GlcNAc for band 1 and β-GlcNAc-(1→2)-α-Man-(1-3)β-GlcNAc-(1→4)[β-GlcNAc-(1→2)-α-Man-(1-6)]β-Man-(1→4)GlcNAc for band 2 (18).

The one-dimensional NMR spectrum for urine of an affected patient (Fig. 5H) showed singlets between 2.02 and 2.08 ppm. The singlet with the highest intensity (2.06 ppm) represents a N-acetylated storage compound at a concentration of 77 μmol/mmol of creatinine.

Sialidosis (OMIM 256550). Sialidosis, or mucolipidosis I, is caused by a deficiency of the lysosomal enzyme neuraminidase. The enzyme cleaves terminal α2→3 and α2→6 sialyl linkages of various glycopeptides, oligosaccharides, and gangliosides. High concentrations of sialyloligosaccharides (bound sialic acid) are found in urine of affected patients.

At least six singlet resonances with a concentration >50 μmol/mmol of creatinine were found between 2.0 and 2.1 ppm by one-dimensional 1H-NMR spectroscopic analysis of the urine of our patient (Fig. 5I). The COSY spectrum of the urine showed two cross-peaks: 1.72–2.67 ppm from the α2→6 sialyl linkages and 1.81–2.67 ppm from the α2→3 sialyl linkages (data not shown). The singlet with the highest intensity (2.03 ppm) was present at a concentration of 430 μmol/mmol of creatinine.

Using TLC, we preparatively isolated two oligosaccharides from the urine of an affected patient (Fig. 1, lane 10). We observed two multiplets (1.75 and 2.66 ppm; cross-peak in the COSY spectrum) in the NMR spectra of both isolated compounds. This allowed us to conclude that both isolated compounds have an α2→6 sialyl linkage. In the spectrum for band 1, three N-acetyl singlets resonated at 2.02, 2.03, and 2.06 ppm, deriving from the two GlcNAc units and the acetylneuraminic acid (AcNeu) unit. From its spectrum we could conclude that band 1 probably is α-AcNeu-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)-β-Man-(1→4)-GlcNAc (19).

Band 2 differed in the chemical shift position of one acetyl singlet (2.05 instead of 2.03 ppm) and the presence of an additional signal at 4.11 ppm, which could indicate compound V in the report by Dorland et al. (19).

Canavan disease (OMIM 271900). Canavan disease is a leukodystrophy attributable to a deficiency of aspartoacylase (EC 3.5.1.15) and belongs to the group of neurotransmitter diseases. We analyzed urine from two unrelated patients. The one-dimensional 1H-NMR spectra for body fluid showed the presence of high concentrations of N-acetylaspartic acid [Fig. 5J; urine from case A, 1760 μmol/mmol of creatinine; urine from case B, 1474 μmol/mmol of creatinine; reference values for all ages, 6–36 μmol/mmol of creatinine (20)].

Citrullinemia (OMIM 215700). Deficiency of the urea cycle enzyme argininosuccinate synthase (EC 6.3.4.5) leads to increased concentrations of citrulline and N-acetylcluttruline in the urine (4, 21).

We analyzed two urine samples from a patient with citrullinemia. The urine 1H-NMR spectrum showed citrulline multiplets (concentration, 825 μmol/mmol of creatinine). We assigned the unusually high singlet at 2.03 ppm to N-acetylcluttruline by comparing the chemical shifts with reference spectra in the literature (22). N-Acetylcluttruline is not commercially available. The urinary N-acetylcluttruline concentrations were 441 and 518 μmol/mmol of creatinine (Fig. 5K).

Tyrosinemia type I (OMIM 276700). Tyrosinemia type I is caused by a deficiency of the enzyme fumarylacetoacetate hydrolase (EC 3.7.1.2). High body fluid concentrations of tyrosine, 4-hydroxyphenylacetic acid, 4-hydroxyphenyl-lactic acid, succinylacetone, and methionine are characteristic of affected patients. We analyzed the urine of a 1-year-old boy with this inborn error.

In one-dimensional 1H-NMR spectra of his urine, we observed high concentrations of tyrosine and 4-hydroxy-
Tyrosinemia type II (OMIM 276600). Deficiency of the cytosolic enzyme tyrosine aminotransferase (EC 2.6.1.5) forms the primary defect of tyrosinemia type II. In the liver it leads to high concentrations of tyrosine, 4-hydroxyphenyllactic acid, 4-hydroxyphenylacetic acid, and N-acetyltyrosine in body fluids. Urine from one patient was analyzed. The concentration of N-acetyltyrosine in the urine of an affected patient was 95 μmol/mmol of creatinine (singlet at 1.94 ppm).

In another patient (age, 0.2 years), we observed a much higher concentration of N-acetyltyrosine in the urine (7780 μmol/mmol of creatinine). This patient received parenteral nutrition containing N-acetyltyrosine. In premature infants (gestational age at birth <36 weeks), urinary N-acetyltyrosine may be as high as 781 μmol/mmol of creatinine (23). In addition, N-acetyltyrosine may be increased in some liver diseases, in which it may be observed along with N-acetyltryptophan (Table 1).

Discussion

N-Acetylated compounds are metabolites in many metabolic pathways and therefore may relate to various inborn errors of metabolism. In this study, we gave examples of 13 inherited diseases involving N-acetylated metabolites. At present, a comprehensive analytical technique to detect and identify N-acetylated compounds in body fluids is not available. However, N-acetylaspartic acid, the biochemical hallmark in Canavan disease, is detected by organic acid analysis by gas chromatography–mass spectrometry. Gas chromatography–mass spectrometry can also be used for the accurate diagnosis of tyrosinemia by analysis for all relevant metabolites, including N-acetyltyrosine. TLC is useful in the identification of abnormal N-acetylated oligosaccharides characteristic of some of the lysosomal storage disorders.

NMR spectroscopy of body fluids can provide an overview of N-acetylated compounds. The protons of the N-acetyl group resonate in a specific narrow region of the spectrum. For metabolic screening purposes, we used the highest resonance in the N-acetyl region to identify samples with unusually high concentrations of N-acetylated metabolite (Fig. 3). This approach allowed different N-acetylated metabolites to be quantified in individual samples. For example, in the urine of a young child, the singlet at 1.94 ppm gave the highest concentration, whereas in an adult urine, the highest concentration was for the singlet at 2.04 ppm. As shown in Fig. 3, most patients with inborn errors of metabolism excreted the N-acetylated metabolites typical for their diseases at concentrations >100 μmol/mmol of creatinine. In 10 of the 13 inborn errors of metabolism, this concentration was clearly higher than the upper limit of the reference interval. In all of these cases the combination of one-dimensional spectroscopy and COSY spectra allowed identification of the compound of interest. This led to the correct diagnosis in 9 of the 13 cases. Exceptions included the urines from patients with tyrosinemia type I and II and G_M1- and G_M2-gangliosidosis.

In tyrosinemia type I, the excretion of succinylacetone and succinylacetocetate in urine is the diagnostic hallmark. To date, gas chromatography–mass spectrometry remains the method of choice to determine succinylacetone and the other characteristic metabolites and to diagnose tyrosinemia type I. For similar reasons the same holds true for tyrosinemia type II.

Because of the relatively low urinary concentration of the G_M1- and G_M2-gangliosidosis metabolites (34 and 77 μmol/mmol of creatinine), assignment of these metabolites in urine in affected patients was not possible, and the diagnosis of G_M1- and G_M2-gangliosidosis was missed by 1H-NMR. NMR spectrometers with higher fields (>500 MHz), and spectrometers using the increased sensitivity of CryoProbe technology could probably detect these two inborn errors of metabolism.

Because of pH standardization, assignments of structures based solely on the chemical shift of the N-acetyl protons are insufficient because many acetyl singlets may occur in urine. Therefore, characteristic COSY patterns of model compounds are required to confirm the presence of individual N-acetylated metabolites in a patient’s urine. For example, the acetyl protons of N-acetylaspartic acid and Man(1-4)GlcNAc showed a singlet at the same chemical shift (2.03 ppm) in the one-dimensional spectrum. Two-dimensional COSY NMR experiments showed for each model compound different and characteristic cross-peaks produced by protons elsewhere in the molecule (Table 1). Because of a change in structure, bound N-acetylneuraminic acid can be distinguished from free N-acetylneuraminic acid in two-dimensional COSY 1H-NMR spectra (Table 1). This makes it possible to differentiate patients with isolated neuraminidase deficiency, who excrete bound N-acetylneuraminic acid, from patients with Salla disease or French type sialuria, who excrete excess free N-acetylneuraminic acid in urine (13). However, in regions with a high density of resonances, especially around the two-dimensional COSY diagonal, cross-peaks sometimes show overlap and cannot be used for correct assignments. Other two-dimensional NMR techniques, such as J-resolved spectroscopy and total correlation spectroscopy, can be used to help with further interpretation.
The use of medications can be a pitfall in the quantification of N-acetylated metabolites. The antiepileptic drug Sabril (vigabatrin) is often used in patients suspected to have an inborn error of metabolism. The H-NMR urine spectra of Sabril-treated patients show high Sabril resonances between 2.0 and 2.1 ppm caused by CH$_2$ protons (multiplet). This Sabril multiplet interferes with the N-acetyl resonances. Therefore, correct interpretation and quantification of the N-acetyl region of the H-NMR spectrum is impossible. Some drugs can be converted in the body and is excreted in the urine in various forms. The $^1$H-NMR spectrum of urine may show acetyaminophen (paracetamol; singlet at 2.1 ppm), 4-glucuronosidoacetanilide (singlet at 2.15 ppm), N-acetyl-4-aminophenol sulfate (singlet at 2.17 ppm), and N-acetyl-2-(N-acetyl-l-cysteinyl)-4-aminophenol (24).

In conclusion, we have shown that careful inspection and quantitative interpretation of the N-acetyl region of the $^1$H-NMR spectrum may identify an abnormally high concentration of an N-acetylated metabolite. The technique is uniquely suited for studying patients with inborn errors of metabolism, and our data extend that to diseases involving N-acetylated metabolites. A combination of one-and two-dimensional COSY $^1$H-NMR spectroscopy identified at least nine different inborn errors of metabolism involving N-acetylated metabolites.

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