Determination of Oligosaccharides in Pompe Disease by Electrospray Ionization Tandem Mass Spectrometry

Tina Rozakis,† Steven L. Ramsay,† Phillip D. Whitfield,† Enzo Ranieri,‡ John J. Hopwood,† and Peter J. Meikle†*

Background: The development of therapies for lysosomal storage disorders has created a need for biochemical markers to monitor the efficacy of therapy and methods to quantify these markers in biologic samples. In Pompe disease, the concentration of a tetrasaccharide, consisting of four glucose residues, is reputedly increased in urine and plasma, but faster and more sensitive methods are required for the analysis of this, and other oligosaccharides, from biologic fluids.

Methods: We optimized the derivatization of storage oligosaccharides with 1-phenyl-3-methyl-5-pyrazolone for the measurement, by electrospray ionization tandem mass spectrometry, of oligosaccharide concentrations in urine (n = 6), plasma (n = 11), and dried-blood spots (n = 17) from Pompe-affected individuals. Age-matched control samples of urine (n = 6), plasma (n = 10), plasma (n = 28), and blood spots (n = 369) were also analyzed.

Results: The mean tetrasaccharide concentration was increased in urine from infantile-onset (0.69–12 mmol/mol of creatinine) and adult-onset (0.22–3.0 mmol/mol of creatinine) Pompe individuals compared with age-matched controls. In plasma samples, an increased tetrasaccharide concentration was observed in some infantile patients (up to 22 μmol/L) compared with age-matched controls (mean, 2.2 μmol/L). The method developed was sensitive enough to determine oligosaccharide concentrations in a single 3-mm blood spot, but no differences were observed between blood spots from control and Pompe-affected individuals.

Conclusions: Measurements of oligosaccharide concentrations in urine by this new method have potential application for the diagnosis and monitoring of patients with Pompe disease. Plasma analysis may have limited application for infantile patients, but analysis of blood spots does not discriminate between controls and affected individuals.

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Pompe disease (glycogen storage disease type II) is one of >40 genetic diseases referred to as lysosomal storage disorders. It is characterized by the lysosomal accumulation of glycogen within cells, resulting from the deficiency of the lysosomal enzyme acid α-glucosidase. Pompe disease can present as the infantile-onset form, which is characterized by massive cardiomegaly, macroglossia, progressive muscle weakness, and marked hypotonia, with death occurring within the first 2 years of life. The juvenile- and adult-onset forms are characterized by slower progressive muscular weakness, with death usually occurring from respiratory failure by the end of the second decade after diagnosis in the juvenile-onset form or later in the adult-onset form (1). The heterogeneous presentation of Pompe disease results, at least in part, as a consequence of different mutations in the lysosomal acid α-glucosidase gene, leading to variation in the functional capacity of the mutant enzyme (2, 3).

Pompe disease is reported to have an incidence of 1 in 201 000 births within the Australian population (4). However, recent studies have indicated that the incidence is likely to be greater, at ~1 in 40 000 births, in both the US (5) and the Netherlands (6). Martiniuk et al. (5) predict that many adult cases resulting from milder mutations are not recognized.

Although a definitive treatment for Pompe disease is...
currently unavailable, two main strategies are being proposed: (a) enzyme-replacement therapy, which has shown promising results in animal studies (7–9), is currently undergoing Phase II clinical trial in Pompe patients (10); and (b) gene transfer of the α-glucosidase gene with viral vectors is being pursued, with successful correction of several cell types in vitro being reported (11–14). Targeted disruption of the gene in different mice strains as a means of modulating the disease severity in this model has also been reported (15).

The development of therapies for Pompe disease has highlighted the need for early and rapid diagnosis of the disorder, as well as suitable biochemical markers for monitoring the efficacy of therapy. We have developed diagnostic assays for Pompe disease that enable rapid diagnosis from a 3-mm blood spot on filter paper (16, 17). This noninvasive procedure should facilitate the earlier diagnosis of this disorder. The use of biochemical markers for Pompe disease has not been widely adopted, despite the early characterization in 1974 (18) that the glucose tetramer (Glc1–6Glc1–4Glc1–4Glc) is increased in the urine of Pompe patients. The tetrasaccharide has been suggested to result from amylase digestion of glycogen in circulation and is thought to be a final product of this process (18). The poor uptake of this oligosaccharide as a diagnostic marker for Pompe disease may be partly attributable to its occurrence in a range of other disorders, including glycogen storage diseases types III and VI (19) and Duchenne muscular dystrophy (20); increases in oligosaccharide concentrations may also take place during pregnancy (21). In addition, the majority of analytical methods are complex and time-consuming, involving gas chromatography–tandem mass spectrometry (gas chromatography–MS/MS)3 (19), HPLC (22), or immunoassays (23). In this report, we describe the development of a rapid, sensitive, and accurate determination of the glucose tetrasaccharide and other oligosaccharides from plasma, urine, or dried-blood spots by MS/MS. We have used this method to determine the concentration of these oligosaccharides in Pompe-affected individuals and controls.

### Materials and Methods

#### Patient Samples

All patient and control samples used in this study were either deidentified or obtained with informed consent. Urine samples from adult Pompe patients (n = 5) were obtained through the Acid Maltase Deficiency Association (Australia). The infantile Pompe urine sample was obtained through the National Referral Laboratory for the Diagnosis of Lysosomal, Peroxisomal and Other Genetic Disorders (Adelaide, Australia). Control urine samples (n = 10) were obtained from apparently healthy adults, children, and infants (Table 1).

Plasma samples from infantile (n = 5) and adult (n = 6) individuals with Pompe disease were from specimens submitted to the National Referral Laboratory and from Dr. Otto van Diggelen (Erasmus University, Rotterdam, The Netherlands). Control plasma samples from adult blood donors (n = 20) were provided by the Australian Red Cross Blood Bank. Plasma samples from control infants (n = 8) were obtained from the National Referral Laboratory (Table 2).

Dr. Martina Baethmann (University of Essen, Essen, Germany) provided blood-spot samples from adult (n = 10), juvenile (n = 4), and infantile (n = 3) Pompe patients. Adult control blood spots (n = 20; matched to the plasma samples) were provided by the Australian Red Cross Blood Bank. Newborn control blood spots (n = 349) were provided from routine samples collected between 48 and 72 h after birth by the South Australian Newborn Screening Centre (Table 3).

Control urine, plasma, and blood-spot samples were from both adults (18–62 years) and infants (0–2 years) to provide age-matched data for the Pompe patients. No blood spots from age-matched juveniles were available. Samples were not sex matched. Urine and plasma samples were stored at −20 °C. Dried-blood spots were stored at −20 °C with desiccant.

#### Reagents

1-Phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Tokyo Kasei Kogyo Co. Ltd. Glucose (Glc), maltose (Glc2), maltotriose (Glc3), maltotetraose (Glc4), maltopentaose (Glc5), maltohexaose (Glc6), maltoheptaose (Glc7), and the internal standard methyl lactose, 4-O-(2-O-methyl-β-D-galactopyranosyl)-D-glucopyranose (MeLac), were purchased from Sigma-Aldrich. Acetonitrile, chloroform, and water were of HPLC grade. Ammonia (280 mg/g) and formic acid (900 mL/L) were of analytical grade. C18 solid-phase extraction columns (25 mg) were purchased as individual units or in a 96-microtiter well format from International Sorbent Technology.

#### Preparation of PMP-Methyl lactose and PMP-Oligosaccharides

MeLac (1.0 μmol) or a mixture of Glc1–Glc7 (1.0 μmol each) was dissolved in 100 μL of derivatizing solution (250 mmol/L PMP, 400 mmol/L NH4, pH 9.1) and heated at 70 °C for 120 min. Each solution was acidic with 100 μL of 800 mmol/L formic acid and then extracted three times with CHCl3 (500 μL each) to remove excess PMP. The aqueous phase was lyophilized three times (from water) to remove excess formic acid and NH4COOH. The PMP-MeLac and PMP-oligosaccharide mixture were each reconstituted in 1 mL of 500 mL/L CH3CN–0.25 mL/L formic acid in water and stored at −20 °C for up to 6 months.

2 Nonstandard abbreviations: MS/MS, tandem mass spectrometry; PMP, 1-phenyl-3-methyl-5-pyrazolone; MeLac, methyl lactose; Hex, hexose; and ESI-MS/MS, electrospray ionization MS/MS.
Optimization of derivatization conditions

To optimize the derivatization conditions, four conditions were investigated: time, temperature, pH, and PMP concentration. Derivatization solutions containing various concentrations of PMP (25 to 250 mmol/L) and pH values of 8.6–10.6 were prepared by dissolving PMP into various concentrations of NH₃ (260 mmol/L to 2 mol/L). Oligosaccharide mixtures (containing 1 nmol each of the Glc₁–Glc₇ calibrators) were derivatized in two series of reactions, differing either by time and pH at constant temperature and PMP concentration or by temperature and PMP concentration at constant time and pH. After derivatization, 1 nmol of PMP-MeLac was added to each reaction mixture and the PMP derivatives were isolated.

Table 1. Oligosaccharide concentration in urine from controls and Pompe patients.

<table>
<thead>
<tr>
<th>Age, years</th>
<th>Hexᵃ</th>
<th>Hex₂ᵃ</th>
<th>Hex₃ᵃ</th>
<th>Hex₄ᵃ</th>
<th>Hex₅ᵃ</th>
<th>Hex₆ᵃ</th>
<th>Hex₇ᵃ</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>28</td>
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<td>0.04</td>
<td>0.09</td>
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<td>40</td>
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<td>1.4</td>
<td>0.15</td>
<td>0.15</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>10</td>
<td>6.8</td>
<td>0.31</td>
<td>0.28</td>
<td>0.08</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>10</td>
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<td>0.22</td>
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<td>0.20</td>
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<td>8</td>
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<td>0.98</td>
<td>0.31</td>
<td>0.35</td>
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<tr>
<td>9</td>
<td>0.5</td>
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<td>0.44</td>
<td>0.36</td>
<td>0.25</td>
<td>0.2</td>
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<tr>
<td>10</td>
<td>0.4</td>
<td>450</td>
<td>255</td>
<td>10.6</td>
<td>0.70</td>
<td>0.33</td>
<td>0.34</td>
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<tr>
<td>Mean of adult controlsᵇ</td>
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<td>3.3</td>
<td>0.26</td>
<td>0.22</td>
<td>0.10</td>
<td>0.18</td>
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<tr>
<td>Mean of infant controlsᶜ</td>
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<td>172</td>
<td>140</td>
<td>6.4</td>
<td>0.69</td>
<td>0.32</td>
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Pompe patients

<table>
<thead>
<tr>
<th>Age, years</th>
<th>Hexᵃ</th>
<th>Hex₂ᵃ</th>
<th>Hex₃ᵃ</th>
<th>Hex₄ᵃ</th>
<th>Hex₅ᵃ</th>
<th>Hex₆ᵃ</th>
<th>Hex₇ᵃ</th>
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<tbody>
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<td>14</td>
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<td>0.39</td>
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<td>0.44</td>
<td>1.8</td>
<td>0.39</td>
<td>0.60</td>
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<td>1.8</td>
<td>0.39</td>
<td>0.60</td>
</tr>
<tr>
<td>4</td>
<td>56</td>
<td>13</td>
<td>14</td>
<td>0.46</td>
<td>1.3</td>
<td>0.15</td>
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</tr>
<tr>
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<td>0.3</td>
<td>34</td>
<td>22</td>
<td>3.5</td>
<td>12</td>
<td>4.5</td>
<td>3.4</td>
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</table>

ᵃ Values expressed as mmol/mol creatinine.
ᵇ Mean of controls 1–5.
ᶜ Mean of controls 7–10.
ᵈ Exact age not known.

Table 2. Oligosaccharide concentration in plasma from controls and Pompe disease patients.

<table>
<thead>
<tr>
<th>Age, years</th>
<th>Hex²ᵃ</th>
<th>Hex₃ᵃ</th>
<th>Hex₄ᵃ</th>
<th>Hex₅ᵃ</th>
<th>Hex₆ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult controls (n = 20)ᵇ</td>
<td>47 (18–62)</td>
<td>2.5 (1.6–9.2)</td>
<td>0.92 (0.65–1.6)</td>
<td>0.43 (0.34–0.82)</td>
<td>0.73 (0.57–1.2)</td>
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<td>Adult Pompe patients</td>
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<td></td>
<td></td>
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<td>3.8</td>
<td>0.48</td>
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<tr>
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<tr>
<td>3</td>
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<td>27</td>
<td>5.6</td>
<td>1.1</td>
<td>1.2</td>
<td>0.66</td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>4.3</td>
<td>1.3</td>
<td>0.37</td>
<td>0.72</td>
</tr>
<tr>
<td>6</td>
<td>Adultᶜ</td>
<td>2.1</td>
<td>0.7</td>
<td>0.32</td>
<td>0.61</td>
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<tr>
<td>Infant controls (n = 8)ᵇ</td>
<td>0.8 (0.0–2.0)</td>
<td>8.3 (4.6–28)</td>
<td>2.2 (1.5–3.2)</td>
<td>0.85 (0.76–1.2)</td>
<td>1.2 (0.67–1.8)</td>
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<tr>
<td>Infantile Pompe patients</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>16</td>
<td>1.9</td>
<td>0.94</td>
<td>1.2</td>
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<td>1.0</td>
<td>1.1</td>
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<tr>
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<td>22</td>
<td>7.4</td>
<td>4.7</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>36</td>
<td>4.2</td>
<td>1.9</td>
<td>1.8</td>
</tr>
<tr>
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<td>0.3</td>
<td>36</td>
<td>12</td>
<td>3.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>

ᵃ Values expressed as μmol/L.
ᵇ Values expressed as the median (range).
ᶜ Exact age not known.
and analyzed by MS/MS as described below. The ratio of the signal from each oligosaccharide to the signal from the external standard (PMP-MeLac) was calculated.

**DERIVATIZATION OF OLIGOSACCHARIDES**

After the optimization of derivatization conditions, the following protocol was developed. Samples of oligosaccharide calibrators or plasma (10 μL) were lyophilized before derivatization. Whole-blood samples were dried on filter paper (S&G 903; Schleicher & Schuell), and 3-mm punches were taken and derivatized directly. Each sample was supplemented with 50 μL of derivatizing solution (250 mmol/L PMP, 400 mmol/L NH₃, pH 9.1, containing 100 pmol of MeLac), vortex-mixed vigorously, and then heated in an oven at 70°C for 90 min. Samples were then acidified with a twofold molar excess of formic acid (50 μL) to the MeLac and used to calculate the concentration of each oligosaccharide in each sample.

**CALIBRATION CURVES AND RESPONSE FACTORS**

Calibration curves for Glc₁–Glc₂ were generated for oligosaccharide concentrations of 0.016–8.0 μmol/L. Oligosaccharide mixtures (containing 1.6–800 pmol each of Glc₁–Glc₇) were derivatized as described above and analyzed by MS/MS.

With each set of samples analyzed, calibrators consisting of 100 pmol each of Glc₁–Glc₇ and MeLac were derivatized and analyzed in triplicate. Response factors were then calculated for each oligosaccharide in relation to the MeLac and used to calculate the concentration of individual oligosaccharides in each sample.

**MASS SPECTROMETRY**

We performed mass spectrometric analysis using a PE Sciex API 365 triple-quadrupole mass spectrometer with an ionspray source and LC-Tune/Multiview data system (PE Sciex). We injected samples (20 μL) into the electrospray source with a Gilson 215 autosampler using a carrying solvent of 500 mL/L CH₃CN–0.25 mL/L formic acid in water at a flow rate of 30 μL/min. For all analytes, nitrogen was used as the collision gas at a pressure of 2.7 × 10⁻³ Pa.

**QUANTITATIVE ANALYSIS OF OLIGOSACCHARIDES**

PMP-derivatized oligosaccharide species were identified by precursor scanning for m/z 175 in the positive-ion mode. Collision energy was set between −46 and −134 V in a linear relationship between m/z 500 and 1500. Quantification of oligosaccharides was performed in the multiple-reaction monitoring mode. Ion pairs monitored were m/z 687/175 (MeLac), 511/175 (Hexose (Hex)), 673/175 (Hex₆), 835/175 (Hex₇), 997/175 (Hex₉), 1159/175 (Hex₁₀), 1321/175 (Hex₁₁), and 1483/175 (Hex₁₂). In a single scan, each ion pair was monitored for 100 ms. For each quantitative measurement, continuous scans were made over the injection period and the mean was calculated. Quantification was achieved by relating the peak heights of the PMP-oligosaccharides to the peak height of the PMP-MeLac internal standard.

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**Table 3. Oligosaccharide concentrations in whole blood from controls and Pompe disease patients.**

<table>
<thead>
<tr>
<th></th>
<th>Age, years</th>
<th>Hex₆⁺</th>
<th>Hex₇⁺</th>
<th>Hex₈⁺</th>
<th>Hex₉⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn controls (n = 349)</td>
<td>0.0</td>
<td>16 (5.6–366)</td>
<td>11 (5.2–54)</td>
<td>7.1 (3.0–30)</td>
<td>3.8 (2.0–8.4)</td>
</tr>
<tr>
<td>Adult controls (n = 20)</td>
<td>47 (18–62)</td>
<td>7.4 (5.2–13)</td>
<td>17 (11–31)</td>
<td>11 (7.1–18)</td>
<td>4.5 (3.3–5.7)</td>
</tr>
<tr>
<td>Infantile Pompe patients (n = 3)</td>
<td>0.0 (0.0–0.3)</td>
<td>8.7 (6.5–10)</td>
<td>8.2 (4.7–14)</td>
<td>5.3 (2.7–10)</td>
<td>4.1 (1.8–6.2)</td>
</tr>
<tr>
<td>Juvenile Pompe patients (n = 4)</td>
<td>6.9 (3.8–9.7)</td>
<td>10 (8.7–32)</td>
<td>17 (13–26)</td>
<td>11 (8.7–17)</td>
<td>5.6 (4.8–6.3)</td>
</tr>
<tr>
<td>Adult Pompe patients (n = 10)</td>
<td>49 (32–59)</td>
<td>11 (5.9–32)</td>
<td>20 (9.2–29)</td>
<td>13 (7.0–20)</td>
<td>5.2 (4.8–7.7)</td>
</tr>
</tbody>
</table>

*Values expressed as μmol/L whole blood.
*Values expressed as the median (range).
**Results**

**Optimization of Derivatization Conditions**

*Time and pH.* Derivatization of identical equimolar samples was allowed to proceed for 0–120 min at a pH range of 8.6–10.6 in 250 mmol/L PMP at 70 °C. Recovery of derivatized oligosaccharides increased up to 90 min, after which there was a slight decrease for most oligosaccharides at 120 min (Fig. 1A). Values between pH 8.6 and 9.7 allowed the most efficient derivatization with a substantial decrease in recovery of derivatized oligosaccharides when pH values >10 were used (Fig. 1B).

*Time and pH.* Derivatization of identical equimolar samples was allowed to proceed for 90 min at pH 9.1 in 25–250 mmol/L PMP at temperatures of 25–80 °C. Temperatures >60 °C were less efficient for derivatization, although the range of 60–80 °C showed little variation (Fig. 2A). The efficiency of derivatization decreased sharply as the concentration of PMP was reduced to <125 mmol/L (Fig. 2B).

On the basis of these results, we determined that the optimum derivatization conditions were 250 mmol/L PMP at pH 9.1 for 90 min at 70 °C.

Derivatization of reducing oligosaccharides was >94%, as determined by derivatization and recovery of [3H]mannose on reversed-phase chromatography. After plasma, urine, or whole-blood samples were supplemented with [3H]mannose, recovery of reducing oligosaccharides from biologic samples was 86%, 89%, and 81%, respectively.

PMP-derivatized oligosaccharides were stable for at least 1 year (no degradation detectable by mass spectrometry) when stored in 500 mL/L CH3CN–0.25 mL/L HCOOH in water at −20 °C.

**Calibration Curves and Response Ratios**

The optimized conditions for derivatization were applied to the preparation of calibration curves. Calibration curves for oligosaccharides Glc₁–Glc₇ were linear over the range (0.016–8 μmol/L) with linear regression values ($R^2$) ≥0.998 (Fig. 3). Depending on the oligosaccharide, at a concentration of 0.016 μmol/L, the signal was two- to fourfold higher than background. This equates to 0.32 pmol of each oligosaccharide injected into the mass spectrometer.

Relative-response ratios were determined for each group of samples analyzed. The relative-response ratios did not vary significantly, with mean values (n = 4) of 1.18 (Glc), 0.87 (Glc₂), 0.56 (Glc₃), 0.49 (Glc₄), 0.28 (Glc₅), 0.13 (Glc₆), and 0.06 (Glc₇). The CVs for these ratios were 14% (Glc), 6.5% (Glc₂), 7.2% (Glc₃), 7.7% (Glc₄), 10% (Glc₅), 9.4% (Glc₆), and 11% (Glc₇).

**Accuracy and Precision of the Assay**

The intraassay CVs were determined for both blood-spot (n = 8) and urine (n = 5) samples. In blood-spot analysis, the CVs for the Hex₂ to Hex₅ concentrations were 5.3%,
7.7%, 10%, and 5.9%, respectively, and in urine samples, the CVs for the Hex to Hex7 concentrations were 3.1%, 2.4%, 2.0%, 1.3%, 1.8%, 2.1%, and 3.9%, respectively. Interassay CVs were determined for blood-spot samples on the basis of 8 separate determinations over 8 days; they were determined for urine samples on the basis of 20 separate determinations over 4 days. CVs for the concentrations of Hex2 to Hex5 in blood spots were 15%, 15%, 13%, and 14%, respectively. In urine samples, the interassay CVs for the concentrations of Hex to Hex7 were 10%, 6.1%, 5.3%, 10%, 12%, 12%, and 16%, respectively.

**Mass Spectrometry of Urine Oligosaccharides**

Derivatization of urine samples from either Pompe or α-mannosidosis patients and controls, followed by MS/MS with a precursor scan of m/z 175, provided characteristic spectra for both the Pompe and α-mannosidosis samples (Fig. 4). Each spectrum showed the oligosaccharide profile that is characteristic of the disorder. In Pompe disease, with the exception of the disaccharide Hex2, the most prominent signal was from the glucose tetramer (Hex4). In α-mannosidosis, there is a series of oligosaccharides starting with the trisaccharide GlcNAc-Man2 up to the pentasaccharide GlcNAc-Man4, presumably resulting from the deficiency of the α-mannosidase enzyme. This demonstrates the potential of this analytical technique to identify other oligosaccharide-storing disorders by their characteristic oligosaccharide profiles.

**Oligosaccharide Concentrations in Pompe Patients**

Urine samples from controls (n = 10) and Pompe disease patients (n = 6) were analyzed for the oligosaccharides ranging from a Hex monosaccharide to a Hex heptasaccharide (Hex7; Table 1). It was not possible to determine the type of hexoses or the linkages involved in these oligosaccharides with the mass spectrometer. Infantile controls (<2 years) showed higher oligosaccharide concentrations compared with adult controls. All Pompe patients showed a marked increase of the glucose tetramer (Hex4) in urine relative to the control groups (adult and infantile). Hex3–Hex7 were also substantially increased. The infantile Pompe patient had higher concen-
trations of oligosaccharides than the adult Pompe patients.

Oligosaccharide concentrations (Hex$_2$–Hex$_8$) in plasma samples from Pompe patients and controls were also analyzed (Table 2). The increase of the Hex$_4$ concentrations was much less pronounced in the plasma samples, with only two of six adult Pompe patients and three of five infantile Pompe patients having concentrations above the ranges for the respective controls. Similarly, the larger oligosaccharide, Hex$_6$, was not increased to the same degree as was observed in the urine samples.

Blood-spot samples from control and Pompe patients were also analyzed (Table 3). Blood spots from newborn and adult controls contained similar concentrations of oligosaccharides. Blood spots from infantile, juvenile, and adult Pompe patients had oligosaccharide concentrations that were indistinguishable from the control population. Hex was not analyzed in plasma and blood spots because the concentration was too high and fell outside the linear range of the assay. Hex$_4$ and Hex$_7$ were also not analyzed in these samples because signals were not above background.

**Discussion**

Biochemical monitoring for metabolic disorders has been performed in diseases such as phenylketonuria for many years. However, as new therapies are developed for additional disorders, there is a continuing challenge to identify new markers and develop new technologies to provide early diagnosis and to monitor the efficacy of therapy in these disorders. Pompe disease is one such disorder in which enzyme-replacement therapy is currently being evaluated, and it is clear that the greatest benefits will be obtained if therapy can be commenced early, before disease progression. We have recently reported methods for the rapid noninvasive diagnosis of this disorder from blood spots on filter paper (16, 17). In this report, we describe the use of MS/MS to quantify the oligosaccharide substrates that are stored in patients with Pompe disease. These oligosaccharides have potential application as diagnostic markers, as well as for the monitoring of disease progression and the efficacy of therapy.

Honda et al. (24) were the first to describe the use of PMP to derivatize reducing sugars, leading to the production of strong ultraviolet-absorbing derivatives for analysis by HPLC. Pitt and Gorman (25) described the analysis of PMP-derivatized oligosaccharides by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. More recently, Shen and Perreault (26) described the electrospray ionization MS/MS (ESI-MS/MS) of PMP derivatives of neutral and N-acetylated oligosaccharides. To have application in a diagnostic setting for the monitoring of patients before and during therapy, methods must be developed for the analysis of suitable oligosaccharides from biologic samples. We have made several modifications to the earlier methods, enabling the analysis of oligosaccharides from samples of urine, plasma, or whole blood spots on filter paper. The major limitation of ESI-MS/MS in biologic samples is the high degree of signal suppression resulting from relatively low amounts of salt contamination. A second limitation, particularly in a pediatric setting, is the small sample volumes available for analysis. We have developed a sample derivatization procedure, to minimize the salt contamination, that is also compatible with small sample volumes.

Our initial studies indicated that the degree of signal suppression resulting from derivatization with NaOH–PMP was of the order of 90–95%, even after desalting the PMP-oligosaccharides on reversed-phase cartridges. To overcome this, we replaced NaOH with NH$_3$ in the derivatization of oligosaccharides with PMP and redefined the conditions for oligosaccharides ranging from mono- to heptasaccharide. The derivatization conditions have been optimized for PMP concentration, pH, time, and temperature. Derivatization for 90 min at 70°C was optimal (Figs. 1 and 2), with 250 mmol/L PMP at pH 9.1 providing the most complete derivatization with a rapid preparation. Although lower pH values could be used, this was difficult to achieve in practice. High PMP concentrations at pH 9.1 showed no adverse effects on oligosaccharide derivatization, as shown in the recovery experiments. The removal of NaOH from the previously reported methods of PMP derivatization provided a significant reduction of signal suppression such that it was of the order of 30–70%, depending on the biologic samples used. This represents a 5- to 10-fold increase in signal, which enabled us to accurately quantify oligosaccharides in samples as small as a 3-mm blood spot containing 3.6 µL of whole blood.

Stable-isotope dilution–MS provides the best method for accurate quantification of compounds in biologic samples. Suitable labeled isotopes are not readily available for oligosaccharides, so we chose to use a nonphysiologic oligosaccharide, MeLac, as an internal standard. We generated calibration curves for the mono- to heptasaccharides in relation to the internal standard (Fig. 3) and used these curves to quantify the oligosaccharides in biologic samples. Calibration curves were linear up to 8 µmol/L, but declined above this concentration.

Comparison of the present method with other methods described previously (22, 27) shows a substantial improvement in sensitivity. The limit of detection reported by Peelen et al. (27) for a HPLC-based method with pulsed amperometric detection was 20–50 pmol, depending on the oligosaccharide analyzed. More recently, An et al. (22) reported a HPLC method involving derivatization with butyl-p-aminobenzoate that had a limit of detection of 4.5 pmol. The limit of detection for the method described here is ~10-fold lower (0.32 pmol). This sensitivity is also reflected in the ability of this method to quantify oligosaccharides from a 3-mm blood spot containing 3.6 µL of whole blood. Modification to the method described by An et al. (22), with a 96-well format for the derivati-
zation, solid-phase extraction, and the shorter run times of the tandem mass spectrometer compared with the HPLC method (3 min vs 30 min, respectively), enabled up to 96 samples to be prepared and analyzed within a single day. This has the potential to be applied for mass screening by mass spectrometric analysis of oligosaccharides.

The methodology has been used to determine the concentration of oligosaccharides in urine, plasma, and blood spots from controls and patients with Pompe disease. Table 1 shows the concentration of oligosaccharides in urine. We observed an increase in the concentration of the glucose tetramer in the Pompe patients compared with the respective concentrations in controls (up to 35-fold) with smaller increases in Hexα, Hexαβ, and Hexβ (up to 14-fold). In plasma samples (Table 2), the differentiation between Pompe and control samples was not as defined. Only three of five infantile patients and two of six adult patients had glucose tetramer concentrations increased above the range of values for controls. In addition, the increase in glucose tetramer concentrations was not as great in plasma as observed in urine. Clearly the usefulness of these markers in plasma will be limited. The rapid clearance of glucose tetramer from circulation has been reported previously (28). In that study, total clearance of an 11-mg intravenous injection of a glucose tetramer into a rhesus monkey was observed within 1.5 h. When a larger dose was administered, the clearance was only partial (12%) within the same time-frame, suggesting uptake and metabolism by an unknown mechanism. It is probable, then, that in most adult and some infantile Pompe patients, the clearance rate of the glucose tetramer oligosaccharide from circulation is balanced by the rate of production from the amylase digestion of glycogen in circulation. Consequently, minimal or no increases are observed in the plasma concentrations of the glucose tetramer compared with controls. A higher concentration of glucose tetramer was determined in whole blood compared with plasma (adult control median values of 11 μmol/L and 0.43 μmol/L, respectively), suggesting that a significant component is present in leukocytes and/or erythrocytes. However, we observed no difference between hexose-tetramer concentrations in blood from controls and Pompe-affected individuals (Table 3), suggesting that there was no significant storage of these oligosaccharides within the cellular component of blood.

In conclusion, determination of oligosaccharides in urine by MS/MS has potential application for the diagnosis of Pompe disease and other oligosaccharidurias such as α-mannosidosis. This methodology is suitable as a screening strategy to be followed by more conventional enzymology. Once a diagnosis has been made, this technology will be useful in the evaluation and monitoring of the patient.

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