Profiling of Pentose Phosphate Pathway Intermediates in Blood Spots by Tandem Mass Spectrometry: Application to Transaldolase Deficiency

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Background: Recently, several patients with abnormal polyol profiles in body fluids have been reported, but the origins of these polyols are unknown. We hypothesized that they are derived from sugar phosphate intermediates of the pentose phosphate pathway (PPP), and we developed a semiquantitative method for profiling of pentose phosphate pathway intermediates.

Methods: Sugar phosphates in blood spots were simultaneously analyzed by liquid chromatography–tandem mass spectrometry using an ion-pair-loaded C18 HPLC column. The tandem mass spectrometer was operated in the multiple-reaction monitoring mode. Enzymatically prepared D-[13C6]glucose 6-phosphate was used as internal standard. The method was used to study sugar phosphates abnormalities in a patient affected with a deficiency of transaldolase (TALDO; EC 2.2.1.2).

Results: In control blood spots, dihydroxyacetone phosphate, pentulose 5-phosphates, pentose 5-phosphates, hexose 6-phosphates, and sedoheptulose 7-phosphate were detected. Detection limits ranged from 100 to 500 nmol/L. Glyceraldehyde 3-phosphate and erythrose 4-phosphate were undetectable. Intra- and interassay imprecision (CVs) were 10–17% and 12–21%, respectively. In blood from the TALDO1-deficient patient, sedoheptulose 7-phosphate was increased.

Conclusions: The new method allows investigation of patients in whom a defect in the PPP is suspected. Measurements of sugar phosphate intermediates of the PPP may provide new insights into metabolic defects underlying the accumulating polyols.

Sugar phosphates, such as D-glucose 6-phosphate (glu-6P),3 play a role as intermediary metabolites in several metabolic pathways, including glycolysis, gluconeogenesis, and the pentose phosphate pathway (PPP). In the PPP, glu-6P is first converted into pentose 5-phosphates, which are subsequently recycled to glu-6P by two enzymes: transketolase and transaldolase (TALDO; Fig. 1).

Recently, we described a patient with a deficiency of TALDO (MIM 602063; TALDO1, EC 2.2.1.2) (1); the patient had early-onset liver cirrhosis. Repeated analyses of urine and plasma showed marked increases in the polyols D-arabitol, ribitol, and erythritol. TALDO activity in lymphoblasts and erythrocytes was deficient, and a homozygous deletion of 3 bp was found in the TALDO gene. We hypothesized that the accumulated polyols in this patient were derived from their corresponding sugars and sugar phosphates in the PPP. In addition, we described a patient with a leukoencephalopathy and increased concentrations of the polyols D-arabitol and ribitol in brain and body fluids (2). To date, we have been

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3 Nonstandard abbreviations: glu-6P, D-glucose 6-phosphate; PPP, pentose phosphate pathway; TALDO, transaldolase; LC-MS/MS, liquid chromatography with tandem mass spectrometry; DHAP, dihydroxyacetone phosphate; ery-4P, D-erythrose 4-phosphate; fru-6P, D-fructose 6-phosphate; gly-3P, D,L-glyceraldehyde 3-phosphate; ribo-5P, D-ribose 5-phosphate; ribu-5P, D-ribulose 5-phosphate; sed-7P, D-sedoheptulose 7-phosphate; xylu-5P, D-xylulose 5-phosphate; ACN, acetonitrile; IS, internal standard; and MRM, multiple reaction monitoring (mode).
unable to find the underlying metabolic defect in this patient. A major problem in studying patients with polyol accumulation in general is the limited information available on human polyol metabolism and transport. We expect that measuring sugar phosphate intermediates of the PPP will lead to more insight into the possible metabolic defects of patients who accumulate polyols.

Various methods have been described for the measurement of sugar phosphates (3–6). Measuring basal sugar phosphate concentrations is difficult because the concentrations are very low [in the nmol/g of wet tissue range (7)]. In addition, distinguishing between different sugar phosphates is complicated because of the similarities in weight and structure. We developed a sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method to simultaneously analyze the intracellular sugar phosphates dihydroxyacetone phosphate (DHAP), glyceraldehyde 3-phosphate, erythrose 4-phosphate, ribose 5-phosphate, ribulose 5-phosphate, xylulose 5-phosphate, fructose 6-phosphate, glucose 6-phosphate, and sedoheptulose 7-phosphate in blood spots. The method was applied to blood spots obtained from fasting and nonfasting controls and from the TALDO-deficient patient.

**Materials and Methods**

**CHEMICALS**
The following sugar phosphates were purchased from Sigma-Aldrich: DHAP, d-erythrose 4-phosphate (ery-4P), d-fructose 6-phosphate (fru-6P), d,L-glyceraldehyde 3-phosphate (gly-3P), d-ribose 5-phosphate (ribo-5P), d-ribulose 5-phosphate (ribu-5P), d-sedoheptulose 7-phosphate (sed-7P), and d-xylulose 5-phosphate (xylu-5P). Glu-6P was purchased from Boehringer Ingelheim BV. ATP, the enzyme glucokinase, and d-[13C6]glucose were purchased from Sigma-Aldrich. Octylamine was purchased from Sigma-Aldrich. Acetic acid (>99% purity), acetonitrile (ACN), diethyl ether, and hexane were purchased from Merck, Sharpe & Dohme BV.

**PARTICIPANTS**
Blood spots were prepared from venous blood from 25 nonfasting children (ages, 0–22 years), 29 nonfasting adults (ages, 25–85 years), and 13 fasting adults (ages, 28–64 years). In addition, blood spots from the TALDO-deficient patient at the age of 12 years were used. The blood samples, obtained with informed consent of the participants, were collected into Vacutainer Tubes containing heparin and were directly cooled on ice to repress endogenous enzyme activity. Within 1 h, 10 μL of blood was pipetted onto filter paper (type 903; Schleicher und Schuell) and was dried overnight. The blood spots were stored at −20 °C until further use.

**SYNTHESIS OF d-[13C6]GLUCOSE 6-PHOSPHATE**
To obtain a stable-isotope-labeled internal standard (IS), we prepared d-[13C6]glucose 6-phosphate by enzymatic synthesis. The enzyme glucokinase (EC 2.7.1.2; 50 U/mL) and 50 mmol/L Tris, pHe 9.0, were used to synthesize d-[13C6]glucose 6-phosphate from d-[13C6]glucose. A 300-μL aliquot of 10 mmol/L d-[13C6]glucose solution was mixed with 165.3 mg of ATP in 9.7 mL of 50 mmol/L Tris buffer, pH 9.0, and 100 μL of 50 U/mL glucokinase. The mixture was incubated for 60 min at 30 °C. The concentration of d-[13C6]glucose 6-phosphate was deter-
determined by gas chromatography with nitrogen phosphorus detection, as described previously (1) and by LC-MS/MS. The isotopic purity determined by LC-MS/MS was >98%. The IS-containing solution was diluted in 500 mL/L ACN in water to a concentration of 1 μmol/L d-[13C6]glucose 6-phosphate and used as extraction solution.

**SAMPLE PREPARATION**

Blood spots and blank filter-paper spots were punched out with a 5-mm diameter disc puncher and placed in wells of a 48-well microtiter plate. To each well we added 150 μL of extraction solution containing 1 μmol/L IS and sealed the microtiter plate with adhesive film. Extraction was carried out by shaking the microtiter plate on an orbital shaker for 20 min. After extraction, 75-μL aliquots were transferred to a fresh microtiter plate, which was subsequently centrifuged for 10 min at 1800g at room temperature. After centrifugation, supernatants were transferred to glass vials, which were capped and stored at −20°C until injection. Calibrators (included in each batch of samples) at concentrations of 1, 5, 10, 15, and 20 μmol/L were prepared from a mixture containing 0.1 mmol/L each of DHAP, ery-4P, xylu-5P, ribo-5P, glu-6P, and sed-7P in water. We added 10 μL of calibration solution to blank filter-paper spots, and these were left to dry for 30 min. Calibrators were then processed as described above.

**PREPARATION OF MOBILE PHASES**

For gradient elution, we used a binary solvent. Solvent A consisted of 125 mL/L ACN–water containing 750 mg/L octylammonium acetate, pH 7.5, and solvent B consisted of 500 mL/L ACN–water containing 750 mg/L octylammonium acetate, pH 7.5. Octylamine, added to the mobile phase as octylammonium acetate, served as an ion-pair reagent on the C18 column, allowing chromatographic clean up and separation of some sugar phosphates. The octylammonium acetate was made by carefully adding 0.1 mole of octylamine and 0.1 mole of acetic acid to 100 mL of diethyl ether on ice. This solution was gently stirred and cooled on dry ice to let the salt crystallize. The ether was removed, and the salt was washed twice with 50 mL of hexane.

LC

LC (Perkin-Elmer Series 200 pump) was performed with a 3.9 × 150 mm Symmetry C18 HPLC column (bead size, 5 μm; Waters Chromatography BV). The column was rinsed with solvent A for 3 min to load the column with ion-pairing reagent. The initial composition of the binary solvent was 100% A, followed by a linear gradient to 25% A-75% B in 10 min. Thereafter, the mobile phase composition changed to 100% A for 3 min to reload the column with the ion-pairing reagent. The flow rate was set to 1 mL/min and was split after the analytical column in a ratio of 1/4, producing an inlet flow into the tandem mass spectrometer of 200 μL/min. For each analysis, 7 μL of sample was injected onto the column, and the total assay time was 13 min.

**MS/MS**

The sugar phosphates were detected with use of an API-3000 tandem mass spectrometer (PE-Sciex) equipped with an electrospray source (Turbo Ion Spray) operating in the negative-ion mode. The ion source settings were as follows: ion spray voltage, −2500 V; source temperature, 400°C; nebulizer gas and collision gas at settings 10 and 4 (arbitrary units), respectively. Other MS/MS settings, such as declustering potential and collision cell energy, were optimized for each particular sugar phosphate. The sugar phosphates were detected with the mass spectrometer in multiple-reaction monitoring (MRM) mode. Data were acquired and processed using Analyst®(TM) for Window NT software (Ver. 1.1).

**RESULTS**

**MASS SPECTRA**

Sugar phosphate calibration solutions containing 10 μmol/L gly-3P, DHAP, ery-4P, ribu-5P, xylu-5P, ribo-5P, glu-6P, fru-6P, or sed-7P, respectively, were used to determine LC-MS/MS fragmentation patterns and retention behavior for each sugar phosphate. Shown in Fig. 2 are mass fragmentograms of DHAP, ery-4P, ribo-5P, xylu-5P, glu-6P, [13C6]glucose 6-phosphate (IS), and sed-7P. All of the sugar phosphates generated a m/z = 97 fragment in the collision quadrupole (Q2), which corresponded to the loss of the sugar moiety in the collision cell. Therefore, the transition of the m/z of the intact sugar phosphate in quadrupole 1 (Q1) to fragment m/z = 97 in Q3 was used for MRM analysis. Fru-6P and glu-6P eluted as one peak, as did ribu-5P and xylu-5P. The MRM transition (Q1/Q3) settings for the different sugar phosphates were as follows: m/z = 169/−97 for DHAP/gly-3P; m/z = 199/−97 for ery-4P; m/z = 229/−97 for ribo-5P, ribu-5P, and xylu-5P; m/z = 259/−97 for fru-6P and glu-6P; m/z = 265/−97 for [13C6]glucose 6-phosphate (IS); and m/z = 289/−97 for sed-7P.

**LINEARITY**

We constructed calibration curves for each of the sugar phosphates by measuring blank filter-paper spots to which 10 μL of solutions containing 0.01, 0.05, 0.1, 0.15, and 0.2 nmoles, respectively, of calibrators had been added, producing calibrator concentrations of 1, 5, 10, 15, and 20 μmol/L. All calibration curves had a coefficient of linear correlation (r2) > 0.99 (Fig. 3). No calibration curve for ery-4P could be constructed because the ery-4P signal was not detectable in this low concentration range.

**LIMITS OF DETECTION, PRECISION, AND RECOVERY**

We estimated the limits of detection for sugar phosphates, at a signal-to-noise ratio of 5, using blank filter-paper
spots to which a solution containing 0.01 nmole of calibrator had been added and verifying the peak height of the analyte and the noise in the chromatographic region of the analyte. The detection limits were 100 nmol/L for DHAP, 10 μmol/L for ery-4P and gly-3P, 400 nmol/L for ribo-5P and ribu-5P/xylu-5P, 500 nmol/L for fru-6P/glu-6P, and 100 nmol/L for sed-7P. The validation data of the presented method are listed in Table 1. All validation experiments were performed in blood spots from one individual. The intraassay variation (CV) was 10–17%, and the interassay variation was 12–21% (Table 1). Recovery experiments were performed in blood spots of one individual, to which 0.1 nmole of DHAP, ery-4P, xylu-5P, ribo-5P, glu-6P, or sed-7P was added. Recoveries were 58% for ribo-5P, 166% for xylu-5P, 232% for DHAP, 79% for glu-6P, and 102% for sed-7P, with CVs of 5–17% (Table 1).

VALUES IN HUMAN CONTROLS AND IN TALDO DEFICIENCY

Control blood spots showed sugar phosphate profiles with clearly distinctive signals for DHAP, ribo-5P, ribu-5P/xylu-5P, fru-6P/glu-6P, and sed-7P. No detectable signals for ery-4P and gly-3P were observed. The sugar phosphate ranges in blood spot samples of 25 nonfasting children (ages, 0–22 years) and in blood spot samples of 29 nonfasting adults (ages, 25–85 years) are listed in Table 2; we found no significant differences in ranges between children and adults. Blood samples from a group of adults in the fasted state were compared with samples from adults in the fed state; we found no significant differences in ranges between the fasted and the fed state (Table 2). One-way ANOVA revealed a significantly lower mean concentration of DHAP in the adults in the fasted state ($P < 0.05$) and significantly higher mean concentrations of fru-6P and glu-6P in children ($P < 0.05$).

The sugar phosphate ranges in the patient with a TALDO deficiency are listed in Table 2. A Student $t$-test

<table>
<thead>
<tr>
<th>Table 1. Imprecision and recoveries for the LC-MS/MS method.</th>
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<tr>
<td>Intraassay (n = 8)(^b)</td>
</tr>
<tr>
<td>DHAP</td>
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<tr>
<td>ribo-5P</td>
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<tr>
<td>ribu-5P/xylu-5P</td>
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<tr>
<td>fru-6P/glu-6P</td>
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<td>fru-6P/glu-6P</td>
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<td>sed-7P</td>
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\(^a\) For recovery studies, 0.1 nmole of sugar phosphate was added to blood spot of one individual (n = 8 for both imprecision and recovery assays).

\(^b\) n = 8 replicates of one blood sample measured.
During the last few years, we have identified several patients with increased concentrations of polyols in body fluids (1, 2, 8). The basic defect in one of them was elucidated (1) but remained unknown in the other patients. At least two problems are involved in the study of patients with increases in polyols. The first problem is that little is known about human polyol metabolism and transport, hampering elucidation of the basic enzyme or transporter defects in the patients. The second problem is that the pathophysiology of disease in the patients is not understood. We hypothesize that inborn errors in the PPP may lead to an accumulation of sugar phosphates and subsequently may produce increased concentrations of polyols. The increased concentrations of polyols and sugar phosphates may both be toxic and contribute to tissue damage. To facilitate research on polyol metabolism and abnormalities in the PPP, we developed a method to measure intracellular sugar phosphate concentrations in human blood spots with MS/MS.

A few previous studies have addressed the assessment of sugar phosphate concentrations. Kauffman et al. (3) quantified PPP metabolites in rat brain and liver tissue indirectly by a spectrophotometric assay and demonstrated that sugar phosphates are present in very low concentrations, i.e., in the nmol/g of wet tissue range. Their method, however, was not specific for different sugar phosphates. For example, no differentiation between ribo-5P and sed-7P was obtained. Jensen et al. (4) developed a MS/MS method for neonatal screening for galactosemia based on the presence of increased intracellular concentrations of galactose 1-phosphate. Other studies reported accumulation of ribo-5P in human infant brain (9) and in tumor cells (10).

The aim of our study was the simultaneous quantification of all PPP intermediates in human blood samples. We first used erythrocytes isolated from venous blood samples. Despite deproteinization of the erythrocytes, we found large intraassay variations for the triose phosphates and hexose phosphates, which were ascribed to enzymatic conversion. To avoid in vitro enzymatic conversion of the compounds of interest, we prepared blood spots. The results obtained in these blood spots were more reliable, with intraassay variations (CVs) of 10–15%. Nevertheless, some in vitro enzymatic conversion does take place, as shown by the recoveries of >100% for xylu-5P and glucose 6-phosphate and <100% for ribo-5P and fructose 6-phosphate. We therefore consider our method semiquantitative.

Ion-pair chromatography provides chromatographic clean up of the sugar phosphates by increasing their retention times, thereby increasing the signal intensities of the sugar phosphates. It enabled us to distinguish between ribo-5P/xylu-5P and ribo-5P/xylu-5P and fructose 6-phosphate and fructose 6-phosphate. Distinguishing between ribo-5P and xylu-5P and between fructose 6-phosphate and fructose 6-phosphate is impossible.

The method we developed has, with two exceptions, low limits of detection, 100–500 nmol/L. The limit of detection for xylu-5P and fructose 6-phosphate was much higher (100 μmol/L), which may be caused by the broad peak shape of these compounds under the chromatographic conditions used. Glyceraldehyde 3-phosphate appeared enzymatically converted to DHAP in blood spots, and it is suggested that erythro-4P accumulation of ribulose 5-phosphate and no detectable accumulation of erythro-4P.

### Discussion

During the last few years, we have identified several patients with increased concentrations of polyols in body fluids (1, 2, 8). The basic defect in one of them was elucidated (1) but remained unknown in the other patients. At least two problems are involved in the study of patients with increases in polyols. The first problem is that little is known about human polyol metabolism and transport, hampering elucidation of the basic enzyme or transporter defects in the patients. The second problem is that the pathophysiology of disease in the patients is not understood. We hypothesize that inborn errors in the PPP may lead to an accumulation of sugar phosphates and subsequently may produce increased concentrations of polyols. The increased concentrations of polyols and sugar phosphates may both be toxic and contribute to tissue damage. To facilitate research on polyol metabolism and abnormalities in the PPP, we developed a method to measure intracellular sugar phosphate concentrations in human blood spots with MS/MS.

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### Table 2. Concentrations of sugar phosphates in blood spots from healthy children and adults and a patient with TALDO deficiency.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>DHAP, μmol/L</th>
<th>ribo-5P, μmol/L</th>
<th>ribu-5P/xylu-5P, μmol/L</th>
<th>fru-6P/gl-6P, μmol/L</th>
<th>sed-7P, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children</td>
<td>25</td>
<td>15.6 (4.56)</td>
<td>1.86 (0.79)</td>
<td>2.34 (0.62)</td>
<td>12.0 (4.24)</td>
<td>1.15 (0.66)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4.21–24.1]</td>
<td>[0.83–5.02]</td>
<td>[1.29–3.56]</td>
<td>[5.51–19.9]</td>
<td>[0.49–3.33]</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted</td>
<td>13</td>
<td>9.60 (6.24)</td>
<td>1.58 (1.31)</td>
<td>1.77 (0.74)</td>
<td>7.75 (2.04)</td>
<td>0.89 (0.41)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4.41–26.9]</td>
<td>[0.60–5.46]</td>
<td>[0.99–3.47]</td>
<td>[5.19–11.1]</td>
<td>[0.55–2.15]</td>
</tr>
<tr>
<td>Fed</td>
<td>29</td>
<td>15.7 (5.17)</td>
<td>1.48 (0.57)</td>
<td>2.17 (0.91)</td>
<td>7.61 (3.36)</td>
<td>0.86 (0.38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[3.99–26.1]</td>
<td>[0.71–2.85]</td>
<td>[0.87–5.23]</td>
<td>[2.06–19.7]</td>
<td>[0.35–2.10]</td>
</tr>
<tr>
<td>TALDO-deficient patient</td>
<td>1</td>
<td>1.88 (0.34)</td>
<td>0.82 (0.22)</td>
<td>1.00 (0.21)</td>
<td>4.86 (1.27)</td>
<td>5.19 (0.84)</td>
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<tr>
<td></td>
<td></td>
<td>[0.74–0.98]</td>
<td>[0.28–0.51]</td>
<td>[0.37–0.51]</td>
<td>[0.92–1.47]</td>
<td>[0.57–1.23]</td>
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<tr>
<td></td>
<td></td>
<td>5.81 (1.56)</td>
<td>1.48 (0.38)</td>
<td>2.80 (0.65)</td>
<td>9.01 (1.67)</td>
<td>5.43 (0.53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4.41–26.9]</td>
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- a Concentrations are the mean (SD) and [range].
- b For ribo-5P and xylu-5P and fru-6P and glu-6P, the substrates elute as one peak and cannot be distinguished from each other.
- c Significantly higher mean concentrations of fru-6P and glu-6P (P <0.05) in children vs adults.
- d Significantly lower mean concentration of DHAP (P <0.05) in fasted vs fed adults.
- e Two withdrawals 1 year apart were made from one patient: measured eight times; ’ measured five times.
- f Significantly different concentration in TALDO-deficient patient (P <0.05) vs mean concentration in children.
- g Significantly higher concentration of sed-7P in TALDO-deficient patient (P <0.05) vs control groups.
could be converted to erythrulose 4-phosphate by the enzyme erythrose 4-phosphate isomerase (EC 5.1.3.1).

We established reference values for intermediates of the PPP in human blood spots and found no age dependency. We also found no influence of fasting on the concentrations of PPP intermediates.

The method can be used for the investigation of patients suspected to suffer from a defect in the PPP. The applicability of the method was demonstrated by analyzing sugar phosphates in a blood-spot sample from a patient affected with a TALDO deficiency. This patient has increased concentrations of pentitols (ribitol and D-arabitol), erythritol, and probably sedoheptitol in body fluids. Surprisingly, only the concentration of sed-7P was increased in this patient, and the concentration of DHAP tended to decrease. Because the PPP is linked to glycolysis by the intermediate DHAP, other factors, such as the nutritional state at time of blood collection, may influence the concentration of this substrate. Rib-5P, ribu-5P/xylu-5P, and ery-4P were within reference values. Small increases in ery-4P may, however, remain undetected because of the high limit of detection of this compound.

In conclusion, our method can be used for semiquantitative profiling of intermediates of the PPP. This analysis was valuable in the investigation of patients with a defect in the PPP. We hope to gain more insight into the underlying defects in patients with polyol accumulation of unknown origin by applying this new methodology.

References