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Study of transaldolase deficiency in urine samples by capillary LC-MS/MS

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Abstract

Transaldolase (TAL) is a key enzyme of the pentose phosphate pathway (PPP). TAL deficiency is a newly recognized cause of liver cirrhosis. We have developed an ion-pair LC separation combined with negative ion electrospray MS/MS detection method to assess PPP metabolites in urine samples from TAL-deficient mice. Sedoheptulose 7-phosphate (S7P), C5-polyols D-arabitol and D-ribitol, and 6-phosphogluconate (6PG) levels were markedly increased in urine of TAL-deficient mice with respect to those of wild-type and heterozygote littermates. The detection limits of S7P, D-arabitol, and 6PG were 0.15 ± 0.015 pmol, 3.5 ± 0.41 pmol, and 0.61 ± 0.055 pmol, respectively. The limit of quantitation was 0.4 ± 0.024 nmol/ml for S7P, 1.6 ± 0.11 nmol/ml for 6PG and 10 ± 0.7 nmol/ml for D-arabitol. Additional metabolites, hexose 6-phosphates (m/z 259), D-ribose 5-phosphate and D-xylulose 5-phosphate (m/z 229), D-fructose 1,6-diphosphate (m/z 339), C6-polyols (m/z 181) and GSSG (m/z 611), that have been positively identified in mouse urine, showed similar levels in control and TAL-deficient mice.

Keywords

capillary liquid chromatography; electrospray mass spectrometry; transaldolase; sugar phosphates; polyols; ion-pair chromatography

INTRODUCTION

The pentose phosphate pathway (PPP) is an alternative route for the metabolism of glucose. It has two major functions, generation of NADPH for reductive synthesis and provision of ribose 5-phosphate for nucleic acid biosynthesis.¹ The key components of the pathway are the phosphorylated monosaccharides. Transaldolase (TAL; EC 2.2.1.2) catalyzes the reversible transfer of dihydroxyacetone phosphate from sedoheptulose 7-phosphate (S7P) to glyceraldehydes 3-phosphate (G3P), thus generating fructose 6-phosphate (F6P) and erythrose-4-phosphate (E4P).² The lack of a TAL enzyme has been detected in patients with liver failure.^{2–4} TAL deficiency patient(s) have higher blood levels of glucose 6-phosphate, F6P, S7P² and elevated levels of polyols in brain, cerebral spinal fluids, and urine. The accumulation of C₄–C₆ polyols⁵ has been associated with neurometabolic disorders.⁵ The TAL-deficient patients were born to related parents; thus, deficiencies of enzymes other than TAL may have contributed to their phenotype. We have analyzed PPP metabolite levels in TAL-deficient ‘knock-out’ (KO) mice. Wild-type (Wt) and heterozygote (Het) littermates

were used as controls. The analysis of monosaccharide phosphates is relatively complicated because of their chemical nature, chromatographic/mass-spectrometric behavior and their low concentration (nmol/g wet tissue). They have isomers that are isobars and their mass-spectrometric fragmentation patterns are also similar,⁶ requiring chromatographic separation^{7–9} prior to mass spectrometry.¹⁰ Because of the polar and acidic nature of the phosphorylated sugars⁷ (pKA, approximately 1), the sample preparation of these compounds is challenging, and conventional reversed phase HPLC separation has been ineffective. Measurements of sugar phosphates have been explored using ion-exchange chromatography^{11,12} or β -cyclodextrin bonded phase^{13–15} or ion-pair reversed phase HPLC.^{2,10,16} A highly selective separation of sugar phosphates and nucleotide sugars was achieved by ion-exchange chromatography.^{11,12} The latter instrumental setup required a high concentration of salt in the eluent (100–500 mM Na acetate) that was not compatible with MS detection⁷ or an on-line ion-suppressor or a desalter unit.¹² β -cyclodextrin columns may support separation of sugar-phosphate isomers;^{13–15} however, β -cyclodextrin columns with internal diameter (i.d.) of 1 mm or less have not been commercially available. Ion-pair chromatography provides only a partial separation for sugar phosphate isomers and requires long equilibration times; however, it is compatible with conventional C₁₈ columns.^{2,10,16} Because of its high sensitivity and extreme accuracy, mass spectrometry has become an optimal approach for analysis of clinical samples.^{6,17} Thus, we developed an LC-MS/MS method for fast and accurate determination of PPP metabolites. In addition to detection of elevated S7P, increased levels of 6-phosphogluconic acid (6PG) and C₅-polyols were also noted in the urine of TAL-deficient mice.

EXPERIMENTAL

Chemicals and reagents

The following chemicals were purchased from Sigma–Aldrich (St Louis, MO): acetonitrile, water, and methanol, all of LC-MS grade; 5-thio-D-glucose-6-phosphate (IS, internal standard); S7P; D-arabitol; 6PG; octylamine; acetic acid; diethyl ether and *n*-hexane.

Mouse urine sample processing

Littermate Wt mice expressing normal TAL protein levels (TAL+/+), Het partially TAL-deficient mice expressing half of normal TAL protein levels due to mutation of one chromosomal allele (TAL+/-), and TAL-deficient KO mice expressing no TAL protein and carrying mutation of both chromosomal alleles (TAL-/-) were utilized. Urine was collected at 18–24 weeks of age and stored at –20 °C until processing for LC-MS/MS. 50 μ l of urine was spiked with 6.44 nmol of 5-thio-D-glucose-6-phosphate IS and mixed with 100 μ l of methanol to precipitate salts and the proteins. Following centrifugation at 4 °C for 25 min at 13 000 rpm, the supernatants were filtered through a 0.45 μ m PVDF membrane filter (Whatman, NJ, USA), re-centrifuged, and transferred to a 250 μ l conical polypropylene HPLC autosampler vial, and 1 μ l was injected into the HPLC column.

Mass spectrometry

The quantification of standard solutions and biological extracts was performed by negative ion electrospray LC/MS/MS on a Waters Q-TOF micro-mass spectrometer with a standard Z-spray configuration (Waters, Milford, MA, USA). Instrument control, data acquisition, and analysis were performed using MassLynx software Version 4.0 (Waters, Milford, MA, USA). Instrument settings were as follows: capillary, –2600 V; source temperature, 110 °C. High purity nitrogen was used as desolvation gas at a flow rate of 150 l/h and heated to 250 °C.

The sampling cone voltage value was optimized to 30 V, the extraction cone voltage was set at 0.0 V, and cone gas nitrogen was applied at 60 l/h. First quadrupole settings were as follows: ion energy, 1 V; collision energy, 5 V for MS mode and 10–15 V for MS/MS mode; low mass (LM) resolution 5.0 and high mass (HM) resolution 5.0; RF lens 1 set at 0.9, RF lens 2 set at 8.0. Hexapole collision cell frequency was 550; argon was used as collision gas at 10 psi operating pressure. Quadrupole pressure was 5.45×10^{-5} torr. The time-of-flight (TOF) analyzer parameters were set as follows: acceleration, 200 V; tube lens, 80 V; TOF flight tube, 5630 V; reflectron, 1780 V; pusher, 820; puller, 750; and pusher frequency, 21 276 Hz. L_{eff} (effective length of the flight tube) and V_{eff} (effective voltage applied for TOF analyzer) settings were 1079.98 mm and 5630 V, respectively. The TOF Penning pressure was 1.41×10^{-7} torr. The MCP (multichannel plate) detector was set to 2700 V. The scan time was set to 1 s in a mass range of 78–750 m/z . The resolution was 4870 full-width at half maximum (FWHM) for a peak of IS at 275.0335 m/z with a corresponding mass accuracy better than 100 ppm. The instrument calibration range was 79–1000 m/z using 0.1% phosphoric acid in 50/50 acetonitrile/water as a calibration solution. The L_{eff} value was checked on a weekly basis by injecting leucine enkephaline (m/z 554.26) in 10 ng/ μ l solution. The selected MS setup parameters for the different compounds are C5-polyols, m/z 151 $\rightarrow$ 89 at 15 V collision energy;¹¹ IS and 6PG, m/z 275 $\rightarrow$ 97 at 12 V; and S7P, m/z 289 $\rightarrow$ 97 at 12 V. The monitored fragment for the phosphorylated compounds is a deprotonated phosphoric acid, which is specific for the phosphorylated compounds.

HPLC separation method

The separation system was a Waters CapLC (Waters, Milford, MA, USA) system with 3 individual syringe pumps and an autosampler. The sample loop was a 5- μ l stainless steel loop. The pump system was programmed to a gradient elution as follows. Eluent A was a water/acetonitrile solution at 87.5: 12.5 ratio with 4 mM octylammonium acetate as ion-pair reagent; eluent B was a water/acetonitrile 50: 50 solution with 4 mM octylammonium acetate as ion-pair reagent. The syringe wash solution was the same as for eluent A. The eluent flow rate was 30 μ l/min and the system was programmed as follows: 0–5 min isocratic 0% B, 5–20 min to 90% B, 20–25 min 90% B isocratic, 25–30 min to 0% B, and 30–40 min 0% B isocratic. The analytical column was a 3- μ m particle size C18 Waters Atlantis with a length of 150 mm and i.d. of 1 mm, which was coupled with a 5- μ m particle size Zorbax bonus RP with a length of 30 mm and i.d. of 1 mm. A 0.5- μ m pore size zero dead volume filter was inserted before the column. One μ l sample was injected using a partial loop injector. The retention time for the IS was in the range 15.2–16.05 min and the error was 1.26% relative standard deviation (RSD) ($N = 100$). The samples were kept at 12 °C in the autosampler tray.

Preparation of octylammonium acetate

Octylamine was added to the mobile phase as octylammonium acetate and served as ion-pair reagent, allowing chromatographic separation and clean-up of the column. The octylammonium acetate was made by dissolving 0.1 mol of octylamine (12.9 g) and 0.1 mol of concentrated acetic acid (6 g) in 100 ml of diethyl ether on ethanol/dry ice bath. The cooled solution was constantly mixed with a magnetic stirrer. After the octylammonium acetate salt crystallized, the ether was removed and the solid crystals were washed twice with 50 ml of *n*-hexane. Before preparation of the HPLC eluents, the octylammonium acetate was frozen at –20 °C.

Standard curve and internal standard (IS) preparation

Testing stock solution contained 592 nmol/ml arabitol, 205 nmol/ml 6PG, and 50 nmol/ml S7P dissolved in water. IS stock solution contained 3.22 μ mol/ml 5-thio-D-glucose-6-phosphate in water. 2, 4, 6, 10, 20, 25, 30, and 50 μ l of the testing stock solution was serially

diluted in water/methanol 50: 50 and 2 μ l of IS stock solution was added to each sample before dilution. The standard calibration samples for urine matrix were made by adding 100, 250, 500, 1000 and 2000 pmol of 6PG and 500, 1000, 2500, 5000 and 10 000 pmol of D-arabitol to 50 μ l of urine of Wt mouse and then the samples treated as described under 'Mouse urine sample processing'.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 3.02 software (San Diego, CA). Paired Student's *t*-test was performed for each litter, employing urine of the Wt genotype as baseline.

RESULTS AND DISCUSSION

Chromatographic and mass-spectrometric optimization

Prior to mass-spectrometric analysis, sugar phosphates were separated on a 3.9-mm i.d. LC column² that was not compatible to our LC system, allowing a maximum flow rate of 40 μ l/min. First, we tested a 50-mm long $\times$ 320- μ m i.d. Waters Atlantis C18 column with 3- μ m particle size. There were many problems with this configuration. Owing to a very low outlet flow (5 μ l/min), the nanospray unit of 20- μ m i.d. spray capillary was rapidly clogged because of the nature of the samples and eluents. No pre-column was available for this analytical column. The autosampler injection volume of 200 nl was excessive for the capacity of the column. Dead volumes were high even if we used 50- μ m i.d. fused silica capillaries.¹⁸ Besides these technical issues, the sensitivity was excellent, the resolution was reasonable, and the retention times were shorter than those for a 1-mm i.d. column, which we used later. Owing to these technical difficulties we changed the column for a 1-mm i.d. 150-mm long Waters Atlantis C18 column with same particle size. A guard column was available for this analytical column, which yielded longer lifetime and more reproducible results. We were able to inject 1 μ l of sample onto this column and no clogging occurred with the Z-spray source. However, sensitivity was reduced and the analysis time was 10 min longer as compared to the 320- μ m LC method.¹⁸ In addition to changing the column dimension, we have used an eluent composition different from that described by Huck *et al.*² The higher percentage of the organic eluent (90% B, instead of 75%) gives a better spray stability, especially for capillary LC setup. Using this LC method for the separation of highly acidic sugar phosphates, we experienced an excellent retention. However, no separation was obtained between the C6 sugar phosphates; i.e. G6P and F6P were co-eluting. The polyols were not retained as much as the acidic components and they were eluting at the beginning of the chromatogram, shortly after the column dead volume. The selected mass fragmentograms and mass spectra are shown in Figs 1 and 2, respectively. The sugar phosphates and C5-polyols were detected using an MS/MS method.^{2,19} The source settings (cone voltage, extraction cone, gas flow, and temperature) were optimized for the IS peak; however, the collision energy was optimized for each detected peak (15 V for the C5-polyols, 12 V for the IS, 6PG and S7P), as mentioned above.

Linear range and limit of quantitation

The calibration curves for S7P, 6-PGA, and D-arabitol had a linear correlation ($r^2 > 0.95$), as shown in Fig. 3. The linear range is 3 orders of magnitude for S7P and 2 orders of magnitude for D-arabitol and 6PG. The analyte concentrations measured in urine samples were consistently in the linear range. The limit of detection was calculated at a signal to noise ratio of 5: 1 (peak-to-peak calculation method). The detection limits were 0.15 ± 0.015 pmol for S7P, 0.61 ± 0.055 pmol for 6PG, and 3.5 ± 0.41 pmol for D-arabitol. The LOQs (limit of quantitation) were 0.4 ± 0.024 nmol/ml for S7P, 1.6 ± 0.11 nmol/ml for 6PG, and 10 ± 0.7 nmol/ml for D-arabitol, obtained using a calibration mixture. Since no blank matrix

was available we have checked the matrix effect with addition of standards to urine, as a biological matrix.²⁰ No significant matrix effect was detected (Fig. 3(D) and (E)).

Sugar-phosphate and polyol values for mouse urine samples

For comparison of different genotypes we have studied 11 complete sets of TAL+/+, TAL+/-, and TAL-/- littermates (2 males and 9 females) at 18–24 weeks of age. We have selected the Wt sets (TAL+/+) as a control. We calculated relative amounts of the following components, hexose phosphates (m/z 259 → 97, mainly a mixture of D-G6P and D-F6P²), pentose phosphates (m/z 229 → 97, mainly D-ribose-5-phosphate and D-xylulose 5-phosphate), C6-polyols (m/z 181 → 89) and the C5-polyols (m/z 151 → 89, a mixture of D-arabitol and D-ribitol²¹), 6PG (m/z 275 → 97) and S7P (m/z 289 → 97). The representative chromatograms of each component sorted by genotypes are shown in Fig. 4(A–C). Significant differences in C5-polyols, 6-PGA and S7P were noted between the wild type and the KO as well as between Het and KO genotypes (Fig. 5(A–C)). The Wt and the Het mice had similar levels of C5 polyol, 6-PGA, and S7P. The C5-polyol level was 62% higher in the KO *versus* Wt urine ($P < 0.0001$, Fig. 5(A)), connecting TAL deficiency to other genetic diseases of polyol accumulation.^{2,3} The level of the 6PG was 53% higher in the KO genotypes than in the Wt or Het genotypes ($P < 0.0001$, Fig. 5(B)). The most striking, >40-fold elevation was observed in S7P levels in urine of KO mice as compared to Wt or Het genotypes ($P < 0.0001$, Fig. 5(C)). This result can be related to the previous finding of a sixfold higher blood level of S7P in a TAL-deficient patient.² Additional components, positively identified as hexose 6-phosphates (m/z 259), D-ribose and D-xylulose 5-phosphate (m/z 229), D-fructose 1,6-diphosphate (m/z 339), C6-polyols (m/z 181), and oxidized glutathione (GSSG) (m/z 611), showed similar levels in control and TAL-deficient mice (data not shown).

CONCLUSION

We have positively identified 8 PPP metabolites in mouse urine including S7P, D-ribose 5-phosphate, D-xylulose 5-phosphate, D-arabitol, 6PG, D-fructose 1,6-diphosphate, D-glucose 6-phosphate, and D-F6P by ion-pair RP LC separation and Q-TOF mass spectrometer for detection. This method offers reliable detection of TAL deficiency based on a 42 ± 3 -fold elevation of S7P in urine of KO mice, as compared to urines of Het and Wt mice from a total of 11 litters. Urine analysis will not distinguish Het carriers from Wt mice. The KO urine samples also contained significantly higher levels of C5-polyols and 6-phosphogluconate. In the blood of TAL-deficient patients, S7P was increased six- to sevenfold and no elevation of C5-polyols and 6PG was noted, suggesting that deficiency of TAL may have been incomplete or mutations in genes other than TAL may have exerted compensatory effects in the patient.

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Figure 1.

MS/MS chromatogram of a standard solution. (A) is the trace of the S7P, (B) is the trace of the IS and 6PG and (C) shows the trace of the D-arabitol. The following amounts were injected onto the HPLC column S7P: 12.5 pmol, IS: 64.4 pmol, 6-PG: 51.25 pmol and D-arabitol: 148 pmol.

Figure 2. MS/MS spectra of the standards (A) is the 12.5 pmol of S7P, (B) is 51.25 pmol 6PG and (C) is the 148 pmol of D-arabital. The instrumental settings are described in the 'Experimental' section.

Figure 3.

Calibration curves for sedoheptulose-7-phosphate, D-arabitol, and 6-phosphogluconate. Calibration for sedoheptulose-7-phosphate was performed in water (panel (A)) while calibration curves for D-arabitol and 6-phosphogluconate were performed in water (panels (B) and (D)) and urine of wild-type control mice (panels (C) and (E)). Panel (A), calibration for sedoheptulose-7-phosphate in water: methanol 1: 1 solution: $a = 0.000417$; $b = 0.02846$ and $r^2 = 0.99$. Panel (B), calibration for D-arabitol in water: methanol 1: 1 solution: $y = a + bx$ where $a = -0.0041$; $b = 0.0039$ and $r^2 = 0.998$. Panel (C), calibration for 6PG in water: methanol 1: 1 solution, $a = -0.0036$; $b = 0.00374$ and $r^2 = 0.989$. Panel (D), lower diagram: calibration for D-arabitol in water: methanol 1: 1 solution: $y = a + bx$ where $a = -0.0041$; $b = 0.0039$ and $r^2 = 0.998$; upper diagram: calibration for D-arabitol in urine matrix: $a_2 = 0.402$; $b_2 = 0.0042$ and $r^2 = 0.95$. Panel (E), lower diagram: calibration for 6PG in water: methanol 1: 1 solution, $a = -0.0036$; $b = 0.00374$ and $r^2 = 0.989$; upper diagram: calibration for 6PG in urine matrix, $a_2 = 0.272$; $b_2 = 0.0035$ and $r^2 = 0.95$.

Figure 4. Representative MS/MS chromatograms of mouse urine samples collected from different genotypes of littermates. (A): C5-polyol (m/z 151 $\rightarrow$ 89), (B): 6PG and the IS (m/z 275 $\rightarrow$ 97) and (C): S7P (m/z 289 $\rightarrow$ 97).

Figure 5.

Analysis of C5-polyol (panel (A)), 6PG (panel (B)), and S7P levels (panel (C)) in urine of 16 KO, 16 Het, and 13 Wt littermates. *P* values indicate significant differences between KO and Wt littermates. The mean standard errors as mentioned in the figure are the following: C5-polyol – Wt: 48.76, Het: 30.5, KO: 34.8; 6PG – Wt: 9.2, Het: 9.3, KO: 11.7; S7P – Wt: 6.88, Het: 12.05, KO: 280.5.