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Quantification of sugar phosphate intermediates of the pentose phosphate pathway by LC–MS/MS: application to two new inherited defects of metabolism

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Abstract

We describe a liquid chromatography tandem mass spectrometry (LC–MS/MS) method to quantify pentose phosphate pathway intermediates (triose-3-phosphates, tetrose-4-phosphate, pentose-5-phosphate, pentulose-5-phosphates, hexose-6-phosphates and sedoheptulose-7-phosphate (sed-7P)) in bloodspots, fibroblasts and lymphoblasts. Liquid chromatography was performed using an ion pair loaded C_{18} HPLC column and detection of the sugar phosphates was carried out by tandem mass spectrometry using an electron ion spray source operating in the negative mode and multiple reaction monitoring. Reference values for the pentose phosphate pathway intermediates in blood spots, fibroblasts and lymphoblasts were established. The method was applied to cells from patients affected with a deficiency of transaldolase. The transaldolase-deficient cells showed an increased concentration of sedoheptulose-7-phosphate. (Bloodspots: 5.19 and 5.43 μmol/L [0.49–3.33 μmol/L]; fibroblasts 7.43 and 26.46 μmol/mg protein [0.31–1.14 μmol/mg protein]; lymphoblasts 16.03 μmol/mg protein [0.61–2.09 μmol/mg protein].) The method was also applied to study enzymes of the pentose phosphate pathway by incubating fibroblasts or lymphoblasts homogenates with ribose-5-phosphate or 6-phosphogluconate and the subsequent analysis of the formed sugar phosphates.

Keywords: Sugar phosphate; Pentose phosphate pathway; LC–MS/MS

1. Introduction

The pentose phosphate pathway (PPP) is a series of interconversions of sugar phosphates. The PPP has two functions: the generation of NADPH for reductive syntheses and oxidative stress responses within cells (Fig. 1), and the formation of ribose residues for nucleotide and nucleic acid biosynthesis. In the irreversible part of the PPP, glucose-6-phosphate (glu-6P) is converted, in three steps, into ribulose-5-phosphate (ribu-5P). Subsequently, the reversible part of the PPP starts with the action of two enzymes: ribulose-5-phosphate epimerase and ribose-5-phosphate isomerase (RPI). These two C5-sugar phosphates are then further metabolized by the enzymes transketolase and transaldolase (TALDO), resulting in the formation of erythrose-4-phosphate (ery-4P) and fructose-6-phosphate (fru-6P). In recent years, we described two new inherited metabolic defects in the PPP: RPI deficiency and TALDO deficiency [1,2]. Patients affected with these defects were found to have elevated con-

Abbreviations: PPP, pentose phosphate pathway; TALDO, transaldolase; RPI, ribose-5-phosphate isomerase; glu-6P, D-glucose-6-phosphate; 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; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; P/S, penicillin and streptomycin; IS, internal standard; MRM, multiple reaction monitoring

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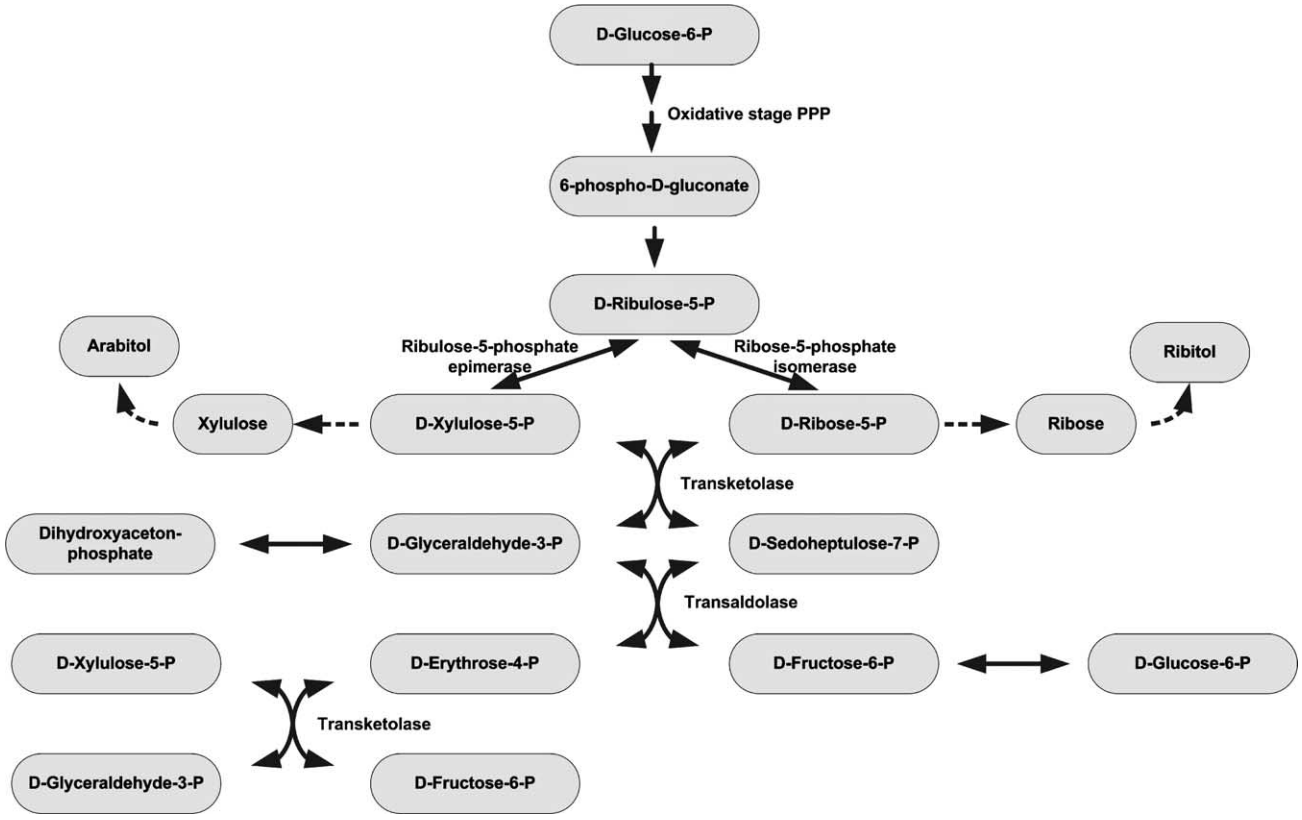

Fig. 1. Schematic representation of the pentose phosphate pathway (PPP) and the presumed reactions leading to formations of pentitols (dashed arrows).

centrations of sugar alcohols, or polyols, in body fluids. The origins of these polyols, mainly D-arabitol and ribitol, are not known.

We hypothesized that intracellular sugar phosphate concentrations are altered in case of a metabolic defect in the PPP, causing the accumulation of the corresponding polyols. This hypothesis prompted us to develop a method for the analysis of intracellular sugar phosphate concentrations.

Various previous studies have addressed the measurement of sugar phosphate levels [3–9]. Measuring basal sugar phosphate concentrations is difficult, because the concentrations are very low (in the nmol/g wet tissue range [10]). In addition, distinction between different sugar phosphates is complicated because of the similarities in weight, charge and structure.

This paper describes our methods for the analysis of sugar phosphate intermediates of the PPP in blood spots and cultured cells. Furthermore, we used the developed method for the analysis of PPP enzyme activities in cultured cells and present results obtained in controls and in patients affected with the two newly discovered defects.

2. Materials and methods

2.1. Chemicals

The following sugar phosphates were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands): D/L-glyceraldehyde-3-phosphate, dihydroxyacetone phosphate (DHAP), D-erythrose 4-phosphate (ery-4P), D-ribose-5-phosphate (ribo-5P), D-sedoheptulose-7-phosphate (sed-7P) and D-xylulose-5-phosphate (xylu-5P). D-glucose-6-phosphate (glu-6P) was purchased from Boehringer Ingelheim BV (Alkmaar, The Netherlands). Perchloric acid (99–100%) and phosphate buffer were purchased from J.T. Baker Chemicals BV (Deventer, The Netherlands). The enzyme glucokinase, $^{13}C_6$-D-glucose, Tris–HCl buffer, thiamine pyrophosphate, 6-phosphogluconate, NAD^+, magnesium chloride and octylamine were purchased from Sigma-Aldrich. Acetic acid (>99% purity), acetonitril (ACN), diethyl ether and hexane were purchased from Merck, Sharpe & Dohme BV. As described earlier [11], $^{13}C_6$-D-glucose-6-phosphate serving as internal standard (IS) was prepared by glucokinase using $^{13}C_6$-D-glucose. Cell culture media (Gibco BRL), fetal bovine serum (FBS) (Gibco BRL), Hanks' balanced salt solution (HBSS, 1×) (Gibco BRL), penicillin and streptomycin (P/S) (Gibco BRL) and trypsin-EDTA (10×) (Gibco BRL) were obtained from Life Technologies (Breda, The Netherlands). All media were supplemented with 10% FBS and 1% P/S.

2.2. Blood spot preparation

Blood spots were prepared as described earlier [11] from venous blood. Within 1 h, 10 μL heparin blood was pipetted onto filter-paper (type 903; Schleicher und Schuell) and dried

overnight. The blood spots were stored at −20 °C until further use. The blood spots and blank filter-paper spots were punched out with a 5 mm diameter disc puncher and placed into wells of a 48-microtiter plate. To each well, 150 μL extraction solution, containing 1 μmol/L of IS ($^{13}C_6$-D-glucose-6-phosphate) in 50% ACN, was added and the microtiter plate was sealed 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 1800 × *g* 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 in water were prepared and 10 μL of each calibration solution was added to individual blank filter-paper spots which were left to dry for 30 min. Calibrators were then processed as described above.

2.3. Fibroblasts isolation

Cultured human fibroblasts obtained from nine control subjects without any known defects in metabolism and from two TALDO-deficient individuals, were grown as monolayers in Nunc 75-cm^2 flasks in Ham F-10 medium. When confluent, the cells were washed free of medium with two rinses of HBSS. Fibroblasts were detached by adding 2 mL of trypsine to the culture flasks. Subsequently, the cells were isolated by centrifugation for 6 min at 350 × *g*. The fibroblast pellet was resuspended in 150 μL HBSS and stored at −80 °C until further preparation and protein determination [12]. This cell suspension could be used for either intracellular sugar phosphate analysis, or for enzyme assays.

2.4. Lymphoblasts isolation

Cultured human lymphoblasts obtained from nine subjects without any known defects in metabolism and from 1 TALDO-deficient individual, were maintained in Nunc 175 cm^2 flasks in RPMI 1640 medium. Lymphoblasts were isolated by centrifugation of the complete cell solution for 6 min at 350 × *g*. After discarding the supernatant, the pellet of lymphoblasts was rinsed once with HBSS and resuspended in 1 mL HBSS. The number of cells was counted and the solutions were diluted to a concentration of approximately 3.5×10^7 cells/mL HBSS. Lymphoblast pellets were stored at −80 °C until further preparation and protein determination [12]. This cell suspension could be used for either intracellular sugar phosphate analysis, or for enzyme assays.

2.5. Cell pellet preparation for sugar phosphate analysis

The cell pellets of fibroblasts and lymfoblasts were sonicated for 30 s on ice and subsequently centrifuged for 5 min at 11,000 × *g* at 10 °C. The supernatant was transferred to a new tube. Stopping residual enzyme activity was done by adding 50 μL 2.5% perchloric acid, containing 3 μmol/L of IS to 10 μL cell pellet samples which were then kept at −20 °C for at least 30 min, for deproteinization. Subsequently, 15 μL of 1 mol/L phosphate buffer (pH 11.5) was added to neutralize the solution and the samples were centrifuged for 5 min at 21,000 × *g* at 4 °C. Supernatants were transferred to glass vials, which were capped and stored at −20 °C until injection. Calibrators were included in each batch of samples and consisted of respectively 5, 10, 20, 30, 50 and 100 μmol/L DHAP and glu-6P, and 0.625, 1.25, 2.5, 3.75 and 6.25 μmol/L ribo-5P, xylu-5P and sed-7P. These calibrators were prepared from a mixture of 0.1 mmol/L DHAP and glu-6P and 12.5 μmol/L ribo-5P, xylu-5P and sed-7P in water. Calibrators were processed as described above.

2.6. Enzyme assays

We developed an enzyme assay [1,2] to determine the activities of transketolase, TALDO and RPI in fibroblasts and lymphoblasts. Ribo-5P which is used as the substrate, is rapidly converted into ribu-5P and then in xylu-5P by the enzymes RPI and ribulose-5-phosphate epimerase, respectively. Transketolase converts ribo-5P and xylu-5P into sed-7P and gly-3P, which are further converted by TALDO. The reaction products of the TALDO reaction are ery-4P and fru-6P.

The cell pellets of fibroblasts and lymfoblasts were sonicated for 15 s on ice and subsequently centrifuged for 5 min at 11,000 × *g* at 10 °C. The supernatant was transferred to a new tube and was incubated with ribo-5P in a total volume of 300 μL, in 45 mmol/L Tris–HCl buffer (pH 8.5), 21 mmol/L magnesium chloride, 0.1 mmol/L thiamine pyrophosphate and 4 mmol/L ribo-5P, at 37 °C. 50 μL samples were taken at 0, 30 and 120 min, and the reaction was terminated by the addition of 50 μl 5% perchloric acid containing 5 μmol/L IS. Thereafter, the samples were kept at −20 °C for at least 30 min. To neutralize the solution, 30 μl of 1 mol/L phosphate buffer (pH 11.5) was added and the samples were subsequently centrifuged for 5 min at 21,000 × *g* at 4 °C. The supernatants were transferred to glass vials, which were capped and stored at −20 °C until injection.

An additional enzyme assay was developed to study RPI separately. Fibroblasts or lymphoblasts were incubated with 6-phosphogluconate, which is a substrate for phosphogluconate dehydrogenase and is converted into ribu-5P. Subsequently ribu-5P is converted to ribo-5P by RPI. The incubation was carried out in a total volume of 3 ml, in 100 mmol/L Tris–HCl buffer (pH 8.6), 33 μmol/L of 6-phosphogluconate, 0.2 mmol/L NAD^+ and 5 mmol/L magnesium chloride. After taking 100 μl samples at 0, 30 and 120 min, sample preparation was performed as described above.

2.7. Analysis of sugar phosphates by LC–MS/MS

Liquid chromatography (Perkin-Elmer series 200 pump) was performed using a 3.9 × 150 mm Symmetry C_{18} HPLC column (bead size 5 μm, Waters Chromatography BV, Etten-

Leur, The Netherlands). For gradient elution, a binary solvent was used as described before [11]. Solvent A consisted of 12.5% ACN/water containing 750 mg/L octylammonium acetate (pH 7.5) and solvent B consisted of 50% ACN/water containing 750 mg/L octylammonium acetate (pH 7.5). The column was rinsed with solvent A for 3 min, to load the column with ion-pair. The initial composition of the binary solvent was 100% A, followed by a linear gradient to 25% A and 75% B in 10 min. Thereafter, the mobile phase composition changed to 100% A for 3 min to reload the column with ion-pair. The flow rate was set to 1 mL/min and was split after the analytical column in a ratio of 1:4, resulting in an inlet flow into the tandem mass spectrometer of 200 μL/min; 7 μL of sample was injected onto the column and the total run time was 13 min.

Detection of the sugar phosphates was carried out on an API-3000 tandem mass spectrometer (PE-Sciex) equipped with an electron ion spray source (Turbo Ion Spray) operating in the negative mode. The ion source parameters were as follows: ion spray voltage, −2500 V; source temperature, 400 °C; nebulizer gas and collision gas at setting 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. Detection of the sugar phosphates was performed by multiple reaction monitoring (MRM-mode). The MRM transitions (Q1/Q3) settings for the different sugar phosphates were DHAP/gly-3P: *m/z* −169/−97; ery-4P: *m/z* −199/−97; ribo-5P, ribu-5P and xylu-5P: *m/z* −229/−97; fru-6P and glu-6P: *m/z* −259/−97, $^{13}C_6$-glucose-6-P (IS): *m/z* −265/−97 and sed-7P: *m/z* −289/−97. Data were acquired and processed using Analyst™ for Window NT software (Ver. 1.3.1).

3. Results

3.1. Fragmentation

In Fig. 2 the mass fragmentograms of a control lymfoblast cell line, containing respectively DHAP, ribo-5P, ribu-5P + xylu-5P, glu-6P + fru-6P, $^{13}C_6$-glu-6P (IS) and sed-7P are shown. All sugar phosphates generated an *m/z* −97 fragment in the collision quadrupole (Q2), which corresponds 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 were found to elute as one peak, as well as ribu-5P and xylu-5P.

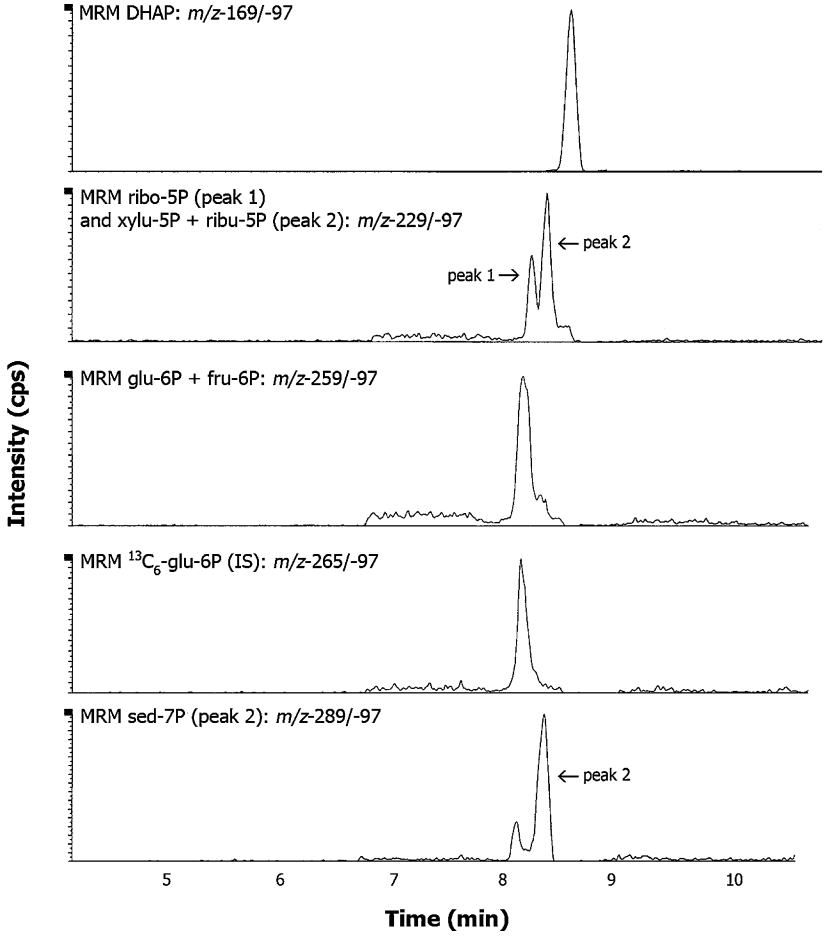

Fig. 2. Mass fragmentogram of a control lymphoblast cell line. MRM transitions are given for each individual sugar phosphate (cps: counts per second).

3.2. Limit of detection

Limits of detection for sugar phosphates in blood spots, at a signal-to-noise ratio = 5, were estimated in blank filter-paper spots spiked with 0.01 nmol calibration standard solution by verifying the peak height of the analyte and the noise in the chromatographic region of the analyte. The detection limits were 0.1 μmol/L for DHAP and sed-7P, 10 μmol/L for ery-4P and gly-3P, 0.4 μmol/L for ribo-5P and ribu-5P/xylu-5P and 0.5 μmol/L for fru-6P/glu-6P.

Detection limits for sugar phosphates in lymphoblasts and fibroblasts, at a signal-to-noise ratio = 5, were estimated with a calibration standard of 5.0 nmol DHAP, ribo-5P, ribu-5P/xylu-5P, 2.5 nmol sed-7P and 0.5 nmol ery-4P and fru-6P/glu-6P. The peak height of the standard sample and the noise of the chromatographic region of the fibroblast or lymphoblast sample were measured. The detection limits were 0.25 μmol/L for DHAP, 1.0 μmol/L for ery-4P, gly-3P and fru-6P/glu-6P and 0.4 μmol/L for ribo-5P, ribu-5P/xylu-5P and sed-7P.

3.3. Intra- and inter-assay variations and recovery

The validation data of the presented method in fibroblasts and lymphoblasts are listed in Table 1 (for the validation data in blood spots see Huck et al. [11]). Determinations of intra- and inter-assay variations and recovery experiments were performed using blood spots from one individual, a fibroblast pool and a lymphoblast pool, respectively. No detectable signals for ery-4P and gly-3P could be observed.

The intra-assay variations (CV) for the sugar phosphates studied were 10–17% in blood spots [11], 3–8% in fibroblasts and 4-15% in lymphoblasts. The inter-assay variation was 12–21% in blood spots [11], 5–10% in fibroblasts and 9–16% in lymphoblasts.

To assess recovery, blood spots from one individual were spiked with 0.1 nmol of DHAP, ery-4P, xylu-5P, ribo-5P, glu-6P and sed-7P, and the fibroblast pool and the lymphoblast pool were spiked with 0.1 nmol of DHAP and glu-6P and 0.125 nmol of ribo-5P, xylu-5P and sed-7P. Recoveries were between 58 and 232% in blood spots with CVs of 5–17% [11], between 95 and 113% in fibroblasts, with CVs of 7–12% and between 85 and 110% in lymphoblasts, with CVs of 7–14% (Table 1).

3.4. References values

Clearly distinctive signals for DHAP, ribo-5P, the combined ribu-5P + xylu-5P and fru-6P + glu-6P peaks, and sed-7P were found in control blood spots, fibroblasts and lymphoblasts. No detectable signals for ery-4P and gly-3P could be observed. The concentrations for sugar phosphates in blood spots samples of 25 non-fasting children and young adults (age 0–22 years) and in blood spot samples of 29 non-fasting and 13 fasting adults (age 25–85 years) were published before together with the concentrations in a TALDO deficient patient [11]. The sugar phosphate ranges in blood spots from the 25 non-fasted children and young adults and the TALDO deficient patient are listed in Table 2. The concentrations for sugar phosphates in fibroblasts and lymphoblasts of nine control subjects are listed in Table 3. A Student's *t*-test revealed significantly higher mean concentrations of DHAP, ribo-5P, ribu-5P and xylu-5P and significantly lower mean concentrations of fru-6P and glu-6P in fibroblasts compared to lymphoblasts ($p < 0.05$).

3.5. TALDO deficient cells

The mean sugar phosphate concentrations in TALDO-deficient fibroblasts and lymphoblasts are also listed in Table 3. A Student's *t*-test revealed significantly increased

Table 1
Imprecision and recoveries for the LC–MS/MS method

	Fibroblasts		Lymphoblasts	
	Imprecision[a] (μmol/L)	Recovery[b,c] (%)	Imprecision[a] (μmol/L)	Recovery[b,c] (%)
Intraasay	$n = 8$	$n = 8$	$n = 6$	$n = 6$
DHAP	44.91 (5)	96 (8)	17.48 (15)	85 (14)
Ribo-5P	5.46 (8)	95 (7)	2.19 (5)	87 (7)
∑Ribu-5P,xylu-5P[d]	8.82 (3)	108 (12)	3.15 (11)	97 (9)
∑Fru-6P,glu-6P[d]	13.97 (3)	110 (7)	24.90 (4)	110 (8)
Sed-7P	8.31 (5)	113 (8)	1.01 (5)	98 (10)
Interassay	$n = 4$	$n = 4$	$n = 5$	$n = 5$
DHAP	41.72 (10)	102 (9)	18.20 (9)	90 (11)
Ribo-5P	5.39 (5)	106 (10)	1.47 (16)	95 (11)
∑Ribu-5P,xylu-5P[d]	7.96 (7)	103 (6)	1.96 (16)	115 (9)
∑Fru-6P,glu-6P[d]	13.48 (7)	102 (6)	27.83 (14)	108 (8)
Sed-7P	7.64 (6)	101 (5)	1.05 (14)	108 (5)

[a] Concentrations are the mean (SD).
[b] For recovery studies, 0.1 nmol of sugar phosphate was added to a pool of fibroblasts and a pool of lymphoblasts, respectively.
[c] Recoveries are mean (SD).
[d] For ribu-5P and xylu-5P and fru-6P and glu-6P, the substrates elute as one peak and cannot be distinguished from each other.

Table 2
Reference values of sugar phosphates in blood spots from children and young adults and TALDO-deficiency

Group	*N*	DHAP[a] (μmol/L)	Ribo-5P[a] (μmol/L)	∑Ribu-5P,xylu-5P[a,b] (μmol/L)	∑Fru-6P,glu-6P[a,b] (μmol/L)	Sed-7P[a] (μmol/L)
Children and young adults	25	15.6 (4.56) [4.21–24.1]	1.86 (0.79) [0.83–5.02]	2.34 (0.62) [1.29–3.56]	12.0 (4.24) [5.51–19.9]	1.15 (0.66) [0.49–3.33]
TALDO-deficiency	1[c]	a: 1.88 (0.34)[*] b: 5.81 (1.56)[*]	a: 0.82 (0.22) b: 1.48 (0.38)	a: 1.00 (0.21)[*] b: 2.80 (0.65)	a: 4.86 (1.27) b: 9.01 (1.67)	a: 5.19 (0.84)[**] b: 5.43 (0.53)[**]

[a] Concentrations are the mean (SD) and [range].
[b] ribu-5P and xylu-5P, and fru-6P and glu-6P; the substrates elute as one peak and cannot be distinguished from each other.
[c] 2 withdrawals with one year in between were made from one patient; a: was measured in eight-fold, b: was measured in five-fold.
[*] Significant change in concentrations in TALDO-deficiency ($p < 0.05$) vs. mean concentrations in children and young adults.
[**] Significant higher concentration of sed-7P in TALDO-deficiency ($p < 0.05$) vs. control groups.

Table 3
Concentrations of sugar phosphates in fibroblasts and lymphoblasts from controls and TALDO-deficient patients

Group	*n*	DHAP[a] (μmol/mg protein)	Ribo-5P[a] (μmol/mg protein)	∑Ribu-5P,xylu-5P[a,b] (μmol/mg protein)	∑Fru-6P + glu-6P[a,b] (μmol/mg protein)	Sed-7P[a] (μmol/mg protein)
Control fibroblasts	9	44.28 (21.52)[c] [16.05–79.85]	6.06 (1.79)[c] [3.54–9.15]	11.95 (5.04)[c] [5.62–14.02]	6.40 (6.44)[d] [1.28–21.36]	0.72 (0.30) [0.31–1.14]
TALDO-deficient fibroblasts[e,f]	2	2.90 (3.36)[f] 7.06 (4.13)[f]	3.63 (1.07) 3.42 (0.77)	6.45 (2.36) 4.26 (1.74)	28.01 (15.71) 13.38 (6.46)	7.43 (1.52)[g] 26.46 (8.66)[g]
Control lymphoblasts	9	17.72 (6.48) [3.23–24.91]	2.21 (0.84) [0.98–3.93]	2.87 (1.19) [1.33–5.18]	23.13 (7.52) [18.16–45.52]	1.03 (0.44) [0.61–2.09]
TALDO-deficient lymphoblasts	1	22.74 (2.08)	3.76 (1.09)	5.06 (1.33)	8.39 (1.58)[h]	16.03 (4.16)[g]

[a] Concentrations are expressed as mean (SD) and [range].
[b] For ribu-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 concentration of DHAP, ribo-5P, ribu-5P and xylu-5P ($p < 0.05$) in fibroblasts vs. lymphoblasts.
[d] Significantly lower mean concentration of fru-6P and glu-6P ($p < 0.05$) in fibroblasts vs. lymphoblasts.
[e] Fibroblasts from two TALDO-deficient patients were measured, both in four replicates.
[f] Significantly lower mean concentration of DHAP in TALDO-deficient fibroblasts ($p < 0.05$) vs. control fibroblasts.
[g] Significantly higher mean concentration of sed-7P in TALDO-deficiency ($p < 0.05$) vs. controls.
[h] Significantly lower mean concentrations of fru-6P and glu-6P in TALDO-deficient lymphoblasts ($p < 0.05$) vs. control lymphoblasts.

concentrations of sed-7P in TALDO-deficient blood spots, fibroblasts and lymphoblasts ($p < 0.05$). Furthermore, significantly decreased concentrations of DHAP ($p < 0.05$) were found in blood spots of the TALDO-deficient patient as compared to healthy children and young adults. We also found a significant decrease in DHAP concentration in TALDO-deficient fibroblasts ($p < 0.05$) and significantly decreased fru-6P and glu-6P concentrations in TALDO-deficient lymphoblasts ($p < 0.05$). No accumulation of pentose phosphates was observed in blood spots, fibroblasts or lymphoblasts.

3.6. Enzyme assays

After 2 hours of incubation with ribose-5-phosphate in control lymphoblasts or fibroblasts formation of ribu-5P + xylu-5P, sed-7P, DHAP and fru-6P +glu-6P was observed indicating that the enzymes RPI, ribulose-5-phosphate epimerase, transketolase and TALDO were active. In lymphoblasts from a TALDO deficient patient no formation of fru-6P + glu-6P was found (Fig. 3A), which is in line with the metabolic defect. Furthermore, sed-7P was significantly higher in the TALDO patient than in controls, suggesting a decreased conversion of this compound in the patient's cells [1]. In RPI deficient cells [2] no formation of ribu-5P + xylu-5P could be observed (Fig. 3B), which is in line with the metabolic defect. After 2 h of incubation with 6-phosphogluconate in RPI deficient cells a decreased formation of ribo-5P could be observed as compared to controls (Fig. 3C) [2].

4. Discussion

Our interest in sugar phosphates was raised by the discovery of two new inborn errors with abnormal profiles of polyols in body fluids. We hypothesize that these abnormal polyols were derived from sugar phosphate intermediates of the PPP and reflected a metabolic defect in this pathway. This idea was substantiated by the results of a newly developed enzyme assay, in which cells were incubated with ribo-5P followed by analysis of sugar phosphate intermediates [1]. The technique used was gas chromatography with nitrogen phosphorous detection, which was suitable for the enzyme assay but failed to detect basal sugar phosphate levels. The metabolic defect in one of the patients was proven to be a deficiency of TALDO. This finding prompted us to develop a sensitive method for analysis of sugar phosphate intermedi-

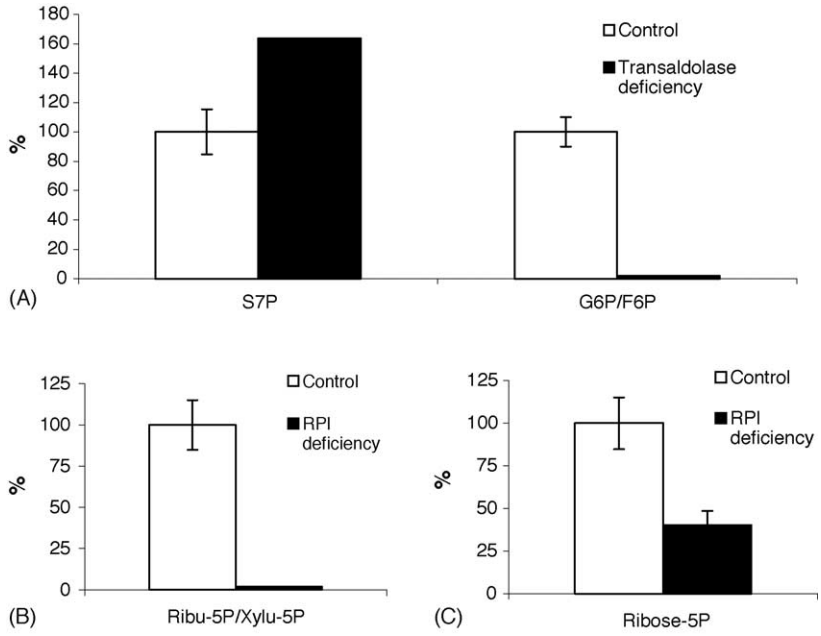

Fig. 3. Enzyme assays. Results are expressed as percentage of the signals obtained in the controls. (A) Formation of sedoheptulose-7-phosphate (S7P) and glucose-6-phosphate + fructose-6-phosphate (G6P/F6P) in lymphoblasts after incubation with ribose-5-phosphate for 120 min. Controls ($n = 5$) and transaldolase deficient patient ($n = 1$, duplicate). (B) Formation of ribulose-5-phosphate + xylulose-5-phosphate (ribu-5P/xylu-5P) in fibroblasts after incubation with ribose-5-phosphate for 120 min. Controls ($n = 3$) and ribose-5-phosphate isomerase deficient patient ($n = 1$). (C) Formation of ribose-5-phosphate (Ribose-5P) in fibroblasts after incubation with 6-phosphogluconate for 120 min. Controls ($n = 3$) and ribose-5-phosphate isomerase deficient patient ($n = 1$).

ates, enabling analysis of these compounds under basal conditions.

Various methods had already been described for the measurement of sugar phosphates. Kauffman et al. [3] have 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: in the nmol/g wet tissue range. However, their method failed to be specific for different sugar phosphates. For example, no differentiation between ribo-5P and sed-7P was obtained. HPLC methods were developed [5,7–9] which were very time consuming. Jensen et al. [4] developed a tandem mass spectrometry method for neonatal screening for galactosemia based on the presence of elevated intracellular concentrations of galactose-1-phosphate.

This paper describes our newly developed method for the analysis of intracellular concentrations of sugar phosphate intermediates by LC–MS/MS. Tandem mass spectroscopy allows the simultaneous detection of the compounds of interest by MRM. Liquid chromatography is applied for the separation of the different compounds. It was shown that ion pair chromatography increased retention times, allowing distinction between pentose-5-phosphate and pentulose-5-phosphates, and improving sensitivity of the measurement. Ribu-5P and xylu-5P could not be quantified independently, as was the same for glu-6P and fru-6P.

First, we developed a method for quantification of sugar phosphate intermediates in blood spots. The results obtained, with intra-assay variations between 10 and 17% seemed solid. Nevertheless, some in vitro enzymatic conversion of added sugar phosphates does take place, as shown by the recoveries of >100% for xylu-5P and DHAP, and <100% for ribo-5P and glu-6P.

We adapted the method for analysis of sugar phosphate intermediates in fibroblasts and lymphoblasts. The recoveries for fibroblasts (95–113%) and lymphoblasts (85–110%) were better than the recoveries in blood spots, due to the use of perchloric acid to deproteinize the samples and inactivate enzyme activity. Low intra-assay and inter-assay variations were found in fibroblasts and lymphoblasts, which indicate that the method is solid in these cell types.

The method we developed has, with two exceptions, a good sensitivity, the detection limit ranging from 0.1 to 1.0 μmol/L. The limit of detection for ery-4P and gly-3P in blood spots was found to be higher (10 μmol/L), which is caused by the broad peak shape of these compounds under the chromatographic conditions used.

We established reference values for intermediates of the PPP in control blood spots, fibroblasts and lymphoblasts and found cell type specificity: fibroblasts have higher concentrations of DHAP, ribo-5P, ribu-5P and xylu-5P than lymphoblasts and lower concentrations of fru-6P and glu-6P. This difference suggests a difference in PPP function in both cells. It is thought that the distribution of the PPP in body tissues is consistent with its function. In erythrocytes, mainly the oxidative stage of the PPP is active for the production of NADPH. The non-oxidative stage of the PPP is present in all cells requiring ribose residues [13].

The applicability of the method was demonstrated by analyzing sugar phosphates in blood spots, fibroblasts and lymphoblasts from a patient affected with TALDO-deficiency. Elevated levels of sed-7P were found as compared to controls.

A different applicability of the method was recently described by Zuurbier et al. [14]. This group investigated the role of the PPP in ischemia. Analysis of PPP intermediates in rat heart tissue under normal and ischemic conditions showed an increase of both ribo-5P and ribu-5P + xylu-5P during ischemia.

The method in this paper not only allows detection of sugar phosphates under physiological conditions, but can also be applied for the determination of enzyme activities. Fibroblasts and lymphoblasts were incubated with ribo-5P or 6-phosphogluconate, after which formation of PPP intermediates were analyzed. In lymphoblasts from a transaldolase-deficient patient there was no formation of fru-6P + glu-6P after incubation with ribo-5P, whereas in controls the formation of these compounds was significant. In RPI-deficient cells [2] no formation of ribu-5P + xylu-5P could be observed after incubation with ribo-5P. After incubation with 6-phosphogluconate in RPI-deficient cells a decreased formation of ribo-5P could be observed as compared to controls [2]. We think that the accumulating sugar phosphates may play a role in pathogenesis of the newly discovered defects of the PPP.

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