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Elevated concentrations of sedoheptulose in bloodspots of patients with cystinosis caused by the 57-kb deletion: Implications for diagnostics and neonatal screening

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ABSTRACT

Cystinosis is an autosomal recessive lysosomal storage disease caused by mutations in *CTNS*. The most prevalent *CTNS* mutation is a homozygous 57-kb deletion that also includes an adjacent gene named *SHPK* (*CARKL*), encoding sedoheptulokinase. Patients with this deletion have elevated urinary concentrations of sedoheptulose. Using derivatisation with pentafluorobenzyl hydroxylamine and liquid chromatography–tandem mass spectrometry (LC–MS/MS), we developed a new sensitive method for the quantification of sedoheptulose in dried blood spots. This method can be utilized as a quick screening test to detect cystinosis patients homozygous for the 57-kb deletion in *CTNS*; which is the most common mutation of cystinosis. Sedoheptulose concentrations in the deleted patients were 6 to 23 times above the upper limit for controls. The assessment of sedoheptulose in a bloodspot from a known cystinosis patient homozygous for the 57-kb deletion retrieved from the Dutch neonatal screening program showed that sedoheptulose was already elevated in the neonatal period. There was no overlap in sedoheptulose levels between cystinosis patients homozygous for the 57-kb deletion and cystinosis patients not homozygous for this deletion. Our presented method can be used prior to mutation analysis to detect cystinosis patients homozygous for the 57-kb deletion. We feel that the presented method enables fast (pre)-symptomatic detection of cystinosis patients homozygous for the 57-kb deletion, allowing early treatment.

1. Introduction

Cystinosis (MIM# 219800) is an autosomal recessive lysosomal storage disease in which intracellular cystine accumulates due to impaired transport out of lysosomes [1]. In nephropatic infantile cystinosis, the most common type of cystinosis, renal tubular Fanconi syndrome occurs in the first year of life, with hypophosphatemic rickets, hypokalemia, polyuria, dehydration and growth retardation. In the natural history of the disease, hypothyroidism, photophobia and renal glomerular failure occur by 10 years of age. Biochemically, the diagnosis of cystinosis is made by the measurement of intracellular cystine in polymorphonuclear leukocytes and/or fibroblasts [2]. The diagnosis can be confirmed by molecular genetic studies.

The main treatment of cystinosis, aside from symptomatic therapy, is oral supplementation with cysteamine (Cystagon®). This aminothiol lowers intracellular cystine content by 95% and has proven efficacy in delaying renal glomerular deterioration, enhancing growth and delaying extra-renal complications [3]. Early diagnosis and treatment are beneficial with respect to renal function, growth, thyroid function and photophobia. In the USA, Australia and Europe, increasing numbers of inborn errors are included in newborn screening programs. However, neonatal screening is currently not available for cystinosis. Ideally, cystine concentration should be determined in bloodspots. However, this is rather complicated due to the high plasma concentration of cystine, which dwarfs the contribution of cellular cystine provided by cystinosis leucocytes [4].

Mutations in the cystinosis gene, *CTNS* (MIM* 606272), coding for cystinosin, cause cystinosis [5]. The most common cause of cystinosis is a 57,257-bp deletion, which is present in about 75% of the patients of North European origin in either homozygous or heterozygous form [6,7]. In the Netherlands almost 50% of cystinosis patients are homozygous for this deletion [8] and in Europe and the USA about

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; DBS, dried blood spots; C7, seven-carbon; PFBHA, O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloric acid.

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33%–44% of affected patients [5,9]. This ~57-kb deletion encompasses not only the *CTNS* gene but also the adjacent gene, named *SHPK* (*CARKL*), encoding sedoheptulokinase [10]. Patients with this deletion have elevated urinary concentrations of sedoheptulose, a seven-carbon sugar [10]. We here describe a new method using tandem mass spectrometry for the determination of derivatised seven-carbon (C7) sugars in dried blood spots (DBS), to clinically diagnose cystinosis caused by a 57-kb deletion; this method can be used as a quick detection of the molecular defect before proceeding to mutation analysis or used into newborn screening programs. Previously, we published a LC–MS/MS method for the quantification of C7-sugars in urine [11], but the sensitivity of that method was not adequate for the low concentrations present in DBS.

2. Materials and methods

2.1. Patients and materials

After informed consent of the families, we obtained DBS samples (collected on Schleicher and Schuell filter paper no. 903) from previously diagnosed cystinosis patients with the homozygous 57-kb deletion (n = 16) or other mutations in the *CTNS* gene (n = 23). DBS samples from 28 controls (age 0–17 years) and 10 healthy newborns were used as controls. DBS samples were stored at room temperature for a maximal period of 1 year prior to analysis. Newborn-screening DBS samples from 5 cystinosis patients (1 homozygous and 4 not homozygous for the 57 kb deletion) were retrospectively measured after 2 to 5 years of storage, for the first year at 4 °C and thereafter at room temperature.

Sedoheptulose was prepared as described by isolation from leaves and stalks of the hybrid plant sedum spectabile [10]. The 48-well plates were purchased from Corning Incorporated (New York, NY, USA), methanol and acetonitrile from Merck (Darmstadt, Germany), and hydrochloric acid from VWR (Leicestershire, UK). O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloric acid (PFBHA), $^{13}C_6$-glucose and ammonium formate were purchased from Sigma-Aldrich (St Louis, MA, USA).

2.2. Sample preparation

A 5.5 mm-diameter blood spot punch (approximately 10 µl of blood) was put in a 48-well plate followed by the addition of 150 µl of extraction fluid consisting of 50% methanol, 0.1 M hydrochloric acid and 3 µM $^{13}C_6$-glucose serving as internal standard. After shaking the plate gently for 15 min, 80 µl of the extract was added to 20 µl of aqueous 75 mM PFBHA reagent and incubated for 30 min at 70 °C. 10 µl of 20 µmol/l standard of sedoheptulose (0.2 nmol) or water was dried on filter paper and prepared the same way. The samples were analysed by liquid chromatography tandem mass spectrometry.

2.3. Liquid chromatography tandem mass spectrometry

Liquid chromatography was performed using a Waters Acquity HPLC equipped with a Waters XTerra RP_{18} analytical column (3.9 × 150 mm; 5 µm bead size) using a flow rate of 0.8 ml/min and an injection volume of 10 µl. For gradient elution, solvent A consisted of 10% acetonitrile/water containing 30 mg/l ammonium formate and solvent B of 60% acetonitrile/water containing 30 mg/l ammonium formate.

The initial composition for the binary gradient was 100% A, followed by a linear gradient to 100% B in 2 min. The column was rinsed with 100% B for 3 min. Thereafter the mobile phase was changed to 100% A and equilibrated for 3 min. Total run time of each sample was 8 min. Detection of sedoheptulose was carried out on a 4000 Q Trap tandem mass spectrometer (AB Sciex) equipped with a TurboIonSpray source operating in negative ionization mode at 550 °C. Other settings were: CUR = 10 psi, CAD = 5, IS = −4500, GS1 = 47 psi, GS2 = 12 psi, ihe = ON, DP = −45 V, EP = −9 V, CE = −10 V and CXP = −10 V.

PFB-oxime derivatives were detected in multiple-reaction-monitoring mode (MRM) using transitions for sedoheptulose m/z −404.2 → m/z −149.1 and $^{13}C_6$-glucose m/z −380.2 → m/z −154.1 with 150 ms dwell time.

Data were acquired and processed using Applied Biosystems Analyst 1.4.2 software.

2.4. Validation

Quantification of sedoheptulose was performed using a single point calibration of the peak area ratio of sedoheptulose towards the internal standard, i.e., $^{13}C_6$-glucose. Assessment of intra-assay and inter-assay variations and recovery experiments were performed using DBS made from pooled blood samples spiked with sedoheptulose. Limit of detection for sedoheptulose, at a signal-to-noise ratio = 5, was estimated in a control DBS, by verifying the peak height of the analyte and the noise in the chromatographic region of the analyte. The stability of sedoheptulose was determined in spiked DBS stored for 1, 7, 14, 21 days at room temperature and for 1 or 2 years at 4 °C.

3. Results

The validation data for sedoheptulose with this method is displayed in Table 1. The intra-assay variation from spiked DBS samples was 5.1%, the inter-assay variation was 12% and the recovery 81%. The limit of detection with a signal-to-noise ratio of 5 was 2 pmol corresponding to a DSB concentration of 0.2 µmol/l. There were no significant changes in sedoheptulose concentrations after storage for 1, 7, 14, 21 days at room temperature or 1 or 2 years at 4 °C.

As shown in Fig. 1 sedoheptulose elutes at 2.9 min. Modest separation between two anomeric forms of sedoheptulose (alpha- and beta-furanoses and/or -pyranoses) was obtained. Both peaks were used for quantification. Mannoheptulose, also a C7-sugar, co-elutes with sedoheptulose and also gives two peaks, theoretically obscuring the quantification of sedoheptulose. However the MS/MS response of mannoheptulose is five times lower than for sedoheptulose (data not shown). The LC–MS/MS MRM data shown in panels A and B of the figure were derived from a normal subject and from a cystinosis patient homozygous for the 57-kb deletion.

The concentrations of sedoheptulose in DBS are shown in Table 2 and Fig. 2. The concentrations in controls were below 0.9 µmol/l, while the concentrations in DBS from patients with the 57-kb deletion ranged from 5.4 to 29.5 µmol/l. Already during the neonatal period sedoheptulose is elevated, as shown in the bloodspot from the neonatal screening program of one cystinosis patient homozygous for the 57-kb deletion. Cystinosis patients without or heterozygous for the 57-kb deletion had normal concentrations of sedoheptulose. The sedoheptulose concentrations in children, neonates born at term and premature newborns were identical, indicating no age dependency.

Table 1
Validation results sedoheptulose in DBS.

	Mean (SD)	CV
Intra-assay (n = 9)	3.6 µmol/l (0.2)	5.1%
Inter-assay (n = 12)	12.9 µmol/l (1.5)	12.0%
Recovery (n = 4)	81% (15%)	–
Limit of detection	2 pmol (0.2 µM in DBS)	–

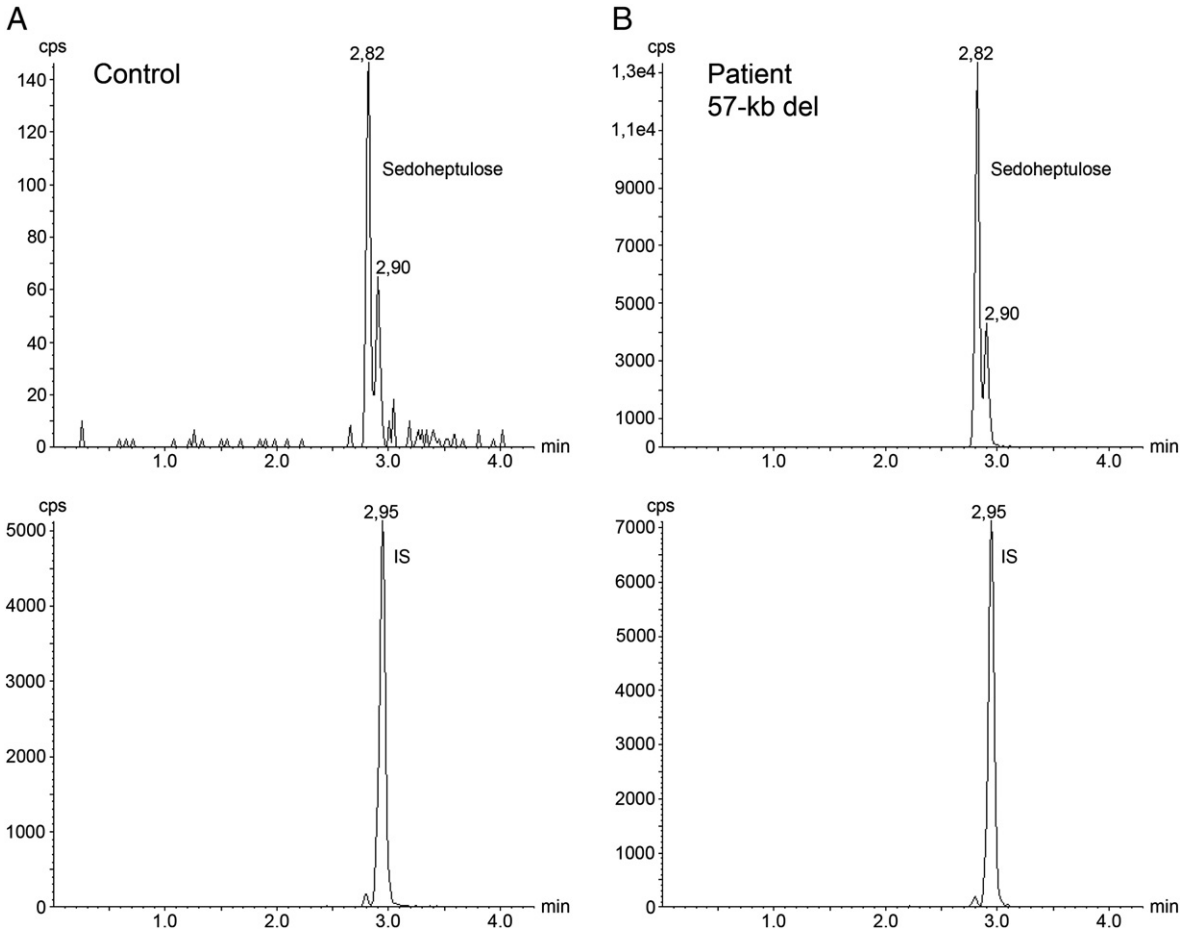

Fig. 1. LC–MS/MS MRM data from PFB-oxime derivatised C7-sugars in DBS extracts from (A) a control subject and (B) a cystinosis patient homozygous for the 57-kb deletion. Upper panel depicts the MRM transition 404.2>149.1 (sedoheptulose) and the lower panel the MRM transition 380.2>154.1 (internal standard (IS), $^{13}C_6$-glucose). The ordinates depict signal intensity (cps); the abscissas denote time (minutes). Please note that the cps scale in the upper panel is different for A and B.

4. Discussion and conclusions

Our new sensitive method for the quantification of sedoheptulose allows detection in DBS corresponding to about 10 µl of blood. Sensitivity, as compared to previously published methods was substantially improved by converting sedoheptulose and the internal standard to their PFB-oxime derivatives. A second beneficial feature of this derivatisation procedure is the increased retention of the sugar-PFB oxime derivatives, allowing simple reversed phase chromatography with traditional mobile phase composition. Because mannoheptulose, a isomer of sedoheptulose, gives a lower MS/MS response compared to sedoheptulose and in urine, normal concentrations of mannoheptulose were found in patients carrying the homozygous 57-kb [10], we assume that in bloodspots of patients carrying the 57-kb deletion only sedoheptulose is elevated and the possible presence of mannoheptulose does not interfere with the analysis.

The advantage of this new method is that it can be applied as a quick screening test to detect cystinosis patients homozygous for the 57-kb deletion. The DBS can be sent by regular mail and only a single drop of blood is required, making it easier accessible than urine

Table 2
Concentrations in µmol/l of sedoheptulose in DBS measured via LC–MS/MS.

Subjects	Mean	SD	Range
Controls (2 mo–17 years) n = 35	0.4	0.2	<0.2–0.8
Cystinosis patients *not* homozygous 57-kb del. n = 23	0.3	0.2	<0.2–0.7
Cystinosis patients homozygous 57-kb del. n = 16	15.2	7.7	5.4–29.5
Neonatal controls (born 29–41 weeks) n = 10	0.4	0.2	<0.2–0.9
Neonatal cystinosis patients *not* homozygous 57-kb del. n = 4	0.2	0.2	<0.2–0.4
Neonatal cystinosis patient homozygous 57-kb del. n = 1	12.0	–	–

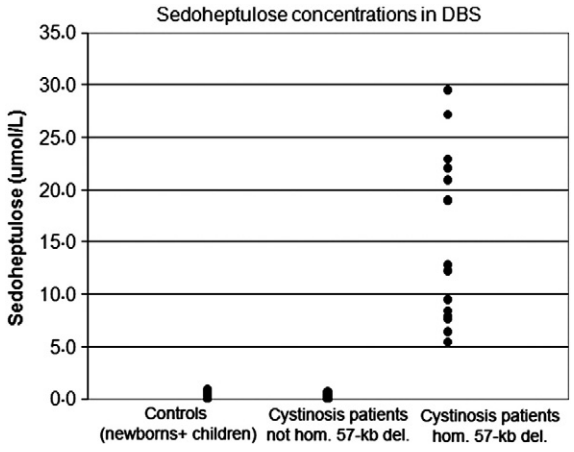

Fig. 2. Scatter plot of sedoheptulose concentrations in DBS measured via LC–MS/MS.

samples. The method can be used prior to molecular mutation analysis to determine if the patient carries the homozygous 57-kb deletion or not. The concentrations of sedoheptulose in the DBS from patients homozygous for the 57-kb deletion were 6 to 32 times above the upper control range. Moreover, there was no overlap between DBS sedoheptulose concentrations from patients homozygous for the deletion and cystinosis patients who did not have the homozygous deletion (Fig. 2). The DBS sedoheptulose concentration did not vary with age or sex, and the concentrations in the neonatal period were identical to those measured in premature newborns. We tested one neonatal DBS sample obtained from a known cystinosis patient carrying the homozygous 57-kb deletion and found that the concentration of sedoheptulose is already clearly elevated in the neonatal period i.e. 13 times the upper control range, underlining that this method can be applied in newborn screening. Cystinosis patients detected by newborn screening will be treated early, thereby preventing early renal glomerular deterioration and other symptoms associated with cystinosis [12].

Before the use in newborn screening programs can be considered, several points require further discussion. First, the assessment of sedoheptulose in DBS will only detect those cases of cystinosis caused by the homozygous 57-kb deletion accounting for approximately 40–50% of nephropathic cystinosis patients in Northern Europe and the United States. If this number is sufficient to warrant implementation in newborn screening programs is a matter of debate. Theoretically, two methods are favourable. The first is the detection of cystine accumulation in DBS. However, such method has not been described to our knowledge. In addition, it is questionable if the number of polymorphonuclear leukocytes in blood spots will be enough to detect their cystine accumulation in cystinosis. The second method would be mutation screening by microarray technology for the most common mutations in the *CTNS* gene. This has previously been proposed by Gahl [13]. Currently, it will be easier to implement a LC–MS/MS method, like our sedoheptulose determination, because new born screening of inborn errors of metabolism is mainly performed by LC–MS/MS, while in the future mutation screening will become applicable in newborn screening programs.

Second, other defects resulting in elevated sedoheptulose, for instance transaldolase deficiency, will be also detected using this method. Transaldolase deficiency is a rare defect in the pentose phosphate pathway [14], with only 16 patients diagnosed to date. No effective treatment has been determined for this disorder, but liver transplantation or treatment with anti-oxidants has been suggested [14]. The assessment of urinary polyols and sugars in urine should then be employed to discriminate between transaldolase deficiency and cystinosis.

Third, for the use of this method in a newborn screening program, further validation of the assay method is necessary, to determine the rate of possible false positives and false negatives. Our preliminary data, however, are promising.

Using LC–MS/MS, we developed a new sensitive method for the quantification of sedoheptulose in DBS, which can be utilized as a quick screening test to detect cystinosis patients homozygous for the 57-kb deletion in *CTNS*.

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