Urine sulfatides and the diagnosis of metachromatic leukodystrophy

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A deficiency of the lysosomal enzyme arylsulfatase A (ASA) causes the lysosomal storage disorder metachromatic leukodystrophy (MLD). The diagnosis of MLD is straightforward in cases with deficient leukocyte or fibroblast ASA activity and a typical clinical history. However, several atypical and late-onset forms of MLD have been described. The diagnosis is also complicated by the high frequency of presumably benign polymorphisms at the ASA gene locus that are associated with markedly diminished in vitro ASA activity. Additional diagnostic tools are needed in the clinically and (or) enzymatically atypical cases. Although analyses of urinary sulfatides have been reported to be helpful in the diagnosis of MLD, previously described methods are complex and incompletely characterized and validated. We developed an improved method for determining urinary sulfatides and applied it to a cohort of individuals with MLD. The sulfatides are extracted from urine, separated from glycerol-based lipids by alkaline hydrolysis, isolated by ion-exchange chromatography, and hydrolyzed to galactosylceramide, which is then perbenzoylated and quantified by HPLC. This assay provides excellent resolution of sulfatides from other lipids and good analytical precision. In addition, the urinary sulfatide concentrations of healthy controls (mean ± SD: 0.16 ± 0.07 nmol/mg creatinine; range: 0.07–0.34; n = 18) are clearly distinguished from those of individuals with MLD (7.6 ± 6.1 nmol/mg creatinine; 1.2–24.2; n = 20).

INDEXING TERMS: arylsulfatase A • sulfatidase • lysosomal storage disease • chromatography, liquid • heritable disorders

Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder characterized by a deficiency of arylsulfatase A (ASA; sulfatidase; EC 3.1.6.8) activity and the lysosomal accumulation of sulfatide (galactosylceramide sulfate).3 Inherited as an autosomal recessive condition, MLD presents in a variety of clinical forms [1]. The most common, and most severe, form of MLD is the late-infantile form, which has an onset at about age 1 year. Juvenile and adult-onset forms also exist. In all forms, the nervous system is affected by a progressive demyelination. Depending on the severity of the biochemical lesion, this results in developmental delays, dementia, ataxia, weakness, progressive spasticity, seizures, and (or) psychiatric abnormalities [1]. Late-onset and atypical forms of MLD have been misdiagnosed as multiple sclerosis, schizophrenia, and other conditions [1].

The diagnosis of MLD is also complicated by the high frequency of two presumably benign alleles of the ASA gene locus, the common ASA pseudodeficiency alleles, which are associated with markedly diminished in vitro ASA activity [2, 3]. An estimated 10–20% of individuals in the general population are heterozygotes for the common ASA pseudodeficiency alleles [4–6] and have reduced in vitro ASA activity, similar to true heterozygotes for MLD. Likewise, individuals who are homozygous for pseudodeficiency alleles have markedly reduced in vitro ASA activity, similar to persons affected with MLD, although their in vivo sulfatidase activity is adequate to maintain sulfatide turnover and thereby prevent the development of symptoms [2, 3, 7, 8].

Yet another complicating factor in the diagnosis of MLD is the existence of a form of MLD that is not caused by a deficiency of ASA but, rather, by a deficiency of an activator protein that works in concert with the enzyme and is required for its intracellular activity [1, 2]. Individuals with the activator deficiency form of MLD show normal in vitro amounts of ASA activity in the commonly used enzyme assays, but their in vivo activity of ASA is pathologically reduced. In such individuals, the exclusive use of leukocyte and fibroblast ASA assays would result in a false-negative result or a failure to diagnose the condition.

Several approaches have been used to differentiate between pseudodeficient and clinically significant ASA-deficient states.

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1 Nonstandard abbreviations: ASA, arylsulfatase A; MLD, metachromatic leukodystrophy; and C/M, chloroform:methanol.
and to ascertain ASA activator deficiency. DNA analysis has been used reliably to detect the pseudodeficiency allele and some MLD mutations [2, 3]. However, the detection of a pseudodeficiency mutation does not preclude the possibility of an additional MLD-causing mutation within the same allele. Individuals who have two mutations, one pseudodeficiency and one deleterious, within the same allele and a second deleterious MLD mutation at the corresponding allele have been reported [9]. Such individuals can easily be misdiagnosed as having a benign compound heterozygote state if the ASA pseudodeficiency mutation is detected and one or both of the deleterious mutations are missed. It is currently not feasible to test for all possible MLD-causing mutations because many of the mutations that have been described are private or uncommon mutations [2, 3], and screening for the most common mutations cannot provide complete assurance that an individual does not carry a deleterious allele.

Another method for differentiating pseudodeficiency and MLD disease states is to analyze the ability of cultured fibroblasts to degrade exogenously added sulfatide [7, 8, 10–12]. Fibroblasts from individuals with MLD have markedly less hydrolysis of exogenously added sulfatide. Pseudodeficient individuals (those with one MLD and one pseudodeficiency allele or two pseudodeficiency alleles) metabolize the sulfatide, albeit at a subnormal rate. However, with this assay, individuals with late-onset forms of MLD are not always easily distinguishable from pseudodeficient individuals [12].

A third way to evaluate complex or atypical cases is to determine urinary sulfatide concentrations. Normal individuals excrete little urinary sulfatide, but individuals with MLD excrete large amounts of sulfatide [1, 13–20]. Thus, an analysis for urinary sulfatide should detect increased sulfatide in the urine of individuals having any clinically significant deficiency of ASA and in urines from individuals with the activator deficiency form of MLD. Determination of the urinary sulfatide concentration is thus an important part of the total evaluation of suspected MLD. However, limited data are available regarding the use of urinary sulfatide analysis for individuals with MLD; urinary sulfatide concentrations have been reported for <25 individuals [14–20]. In addition, previously described methods of urinary sulfatide analysis are incompletely characterized with respect to important clinical chemistry characteristics, and some provide suboptimal resolution of the sulfatides from other lipids [14–20]. Here we describe an improved HPLC method for the analysis of urinary sulfatides and report its application to urine specimens from a large cohort of clinically and enzymatically verified individuals with MLD.

**Materials and Methods**

**Samples**

Aliquots of 10–20 mL of urine were obtained from 18 apparently healthy unaffected individuals of various ages and from 20 individuals with clinically and enzymatically confirmed MLD. Urine samples were stored at −20 °C, and later thawed and mixed thoroughly before use. Enzymatic analysis of ASA activity was performed by the method of Lee-Vaupel and Conzelmann [21].

**Reagents**

Purified bovine brain sulfatide was obtained from Sigma Chemical Co. (St. Louis, MO). The solvents were HPLC-grade and obtained from Fisher Scientific (Pittsburgh, PA). Other chemicals were reagent-grade and also obtained from Fisher Scientific, except for the dry methanolic HCl (from Supelco, Bellefonte, PA), C₁₈ silica (Separyle; Analytichem International, Natick, MA), and DEAE-52 (Whatman, Clifton, NJ).

**Sulfatide Analysis**

The complete method is outlined in Fig. 1.

**Extraction of glycolipids.** Aliquots of 10–20 mL of urine of known creatinine concentration were frozen at −70 °C and then lyophilized in 50-mL conical plastic tubes. The residue was resuspended in 3 mL of 0.1 mol/L KCl, and transferred to 30-mL glass screw-cap tubes. After the addition of methanol (4 mL) and chloroform (8 mL), the solution was vigorously mixed at ambient temperature for at least 2 h. The resulting two phases were separated by centrifugation (400g, 10 min), and the upper phase was discarded. The lower phase was filtered through glass wool and evaporated to dryness under nitrogen.

**Alkaline hydrolysis.** The samples were subjected to mild alkaline hydrolysis by the addition of 1 mL of 0.6 mol/L methanolic NaOH to each sample. After 1 h at ambient temperature, the reaction mixture was neutralized with 1.5 mL of 0.4 mol/L HCl. The sample was then mixed with 1 mL of methanol and 0.5 mL of water, twice passed through a 0.4-mL column of C₁₈ silica (Separyle) that had been prewashed with 2.5 mL of chloroform: methanol [2:1 by vol (C:M 2:1)], C:M (1:1), 65% ethanol, and water. The loaded column was then washed with 3.5 mL of 0.1 mol/L KCl in an equilvolume mixture of methanol and water, followed by 3.5 mL of water, and the lipids were eluted from the column with 5 mL of methanol. The sample was brought to dryness under nitrogen and redissolved in 1 mL of C:M (2:1).

**Isolation of sulfatides.** The sample was then applied to a 2-mL column of DEAE-52 that had been previously equilibrated with 0.8 mol/L methanolic sodium acetate, washed with 8 mL of methanol, and washed with 8 mL of C:M (2:1). Residual sample was rinsed onto the column with two additional 1-mL washes of C:M (2:1). Neutral lipids were eluted from the column with 15 mL of C:M (2:1), after which the sulfatide fraction was eluted with 20 mL of 0.5 mol/L potassium acetate and 0.14 mol/L ammonium hydroxide in C:M (4:1). The sulfatide fraction was brought to dryness and redissolved in 20 mL of methanol:water (1:1 by vol); the redissolved fraction was then applied twice to a 0.4-mL C₁₈ silica column that had been equilibrated by prior washing with 2.5 mL of 0.1 mol/L KCl in an equilvolume solution of methanol and water, followed by 3.5 mL of water. After the column was washed with 2.5 mL of methanol:water (1:1 by vol), the sulfatides were eluted from the column with 5 mL of methanol, and this solution was evaporated to dryness and lyophilized to remove any moisture.
Hydrolysis of sulfate. The sample was subjected to mild acid hydrolysis in 1 mL of dry 0.05 mol/L methanolic HCl at ambient temperature for 16 h, after which 3 mL of chloroform, 0.5 mL of methanol, and 1.1 mL of 2 g/L sodium bicarbonate were added; the two phases were separated by centrifugation, and the upper phase was discarded. The lower phase was rinsed with 2 mL of C:M:0.1 mol/L aqueous KCl (3:48:47, by vol) and then with C:M:water (3:48:47, by vol). The upper phases were discarded, and the lower phase (containing sulfatide-derived galactosylceramide) was brought to dryness and lyophilized.

Perbenzoylation. To each sample was added 0.5 mL of 100 mL/L benzoyl chloride in dry pyridine. After 16 h at 37 °C, the perbenzoylated samples were brought to dryness and redissolved in 3 mL of hexane. The hexane solution was washed with prolonged vigorous mixing five times with 2 mL of 0.4 g/L \( \text{Na}_2\text{CO}_3 \) in methanol:water (4:1 by vol), followed by three washings with methanol:water (4:1). The upper hexane layer was then evaporated to dryness at ambient temperature under nitrogen and dissolved in CCl₄.

HPLC. Aliquots of the perbenzoylated glycolipids in CCl₄ were separated by HPLC on a 500 × 2.1 mm column of Zipax uncoated silica (Rockland Technologies, Gilbertsville, PA) by using a 16-min linear gradient from 10 to 200 mL/L dioxane in hexane at a flow rate of 2 mL/min. The absorbance at 229 nm was monitored, and the data were collected on a Macintosh SE computer with the use of Dynamax software (Rainin, Emeryville, CA).

**ANALYSIS OF DATA**

The amount of sulfatide was calculated by converting the area under the two galactosylceramide peaks into picomoles of glycosphingolipid, as determined from an authentic standard [22]. Correction for losses during the procedure was performed by determining the yield of a concurrently analyzed sulfatide standard of known concentration (5.6 \( \mu \text{mol/L} \)), from which a standard factor (\( f \)) was calculated. For example, for a typical 30% final yield, the standard factor would be 3.3. The concentration of urinary sulfatide (as nanomoles per milligram of creatinine) was calculated as:

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\text{sulfatide measured (nmol)} = \frac{1}{\text{volume of urine (mL)}} \times \frac{1}{\text{creatinine (mg/mL)}} \times f
\]

**Results**

**CHROMATOGRAPHIC RESOLUTION**

Representative chromatograms are shown in Fig. 2. These illustrate the excellent HPLC-based resolution of the sulfatide-
Fig. 2. Representative chromatograms of urinary sulfatides: (top) a typical MLD patient urine, (middle) the sulfatide standard, and (bottom) a healthy control urine.

derived hydroxy and nonhydroxy fatty acid galactosylceramides from the peaks for any other residual glycolipids.

ANALYTICAL PERFORMANCE

Linearity and dynamic range of the assay. Amounts of sulfatide ranging from 50 to 6400 pmol were added to 20 mL of water (final concentrations, 2.5–320 nmol/L) and processed as described in Materials and Methods. The assay results were linear from 100 to 6400 pmol/20 mL (Fig. 3, left). Therefore, the limit of detection of this assay is 100 pmol of sulfatide.

A similar experiment was performed by adding sulfatide standards (1000 to 11 000 pmol) to 20-mL aliquots of a normal control urine and processing as described in Materials and Methods. As shown in Fig. 3 (right), the assay was linear throughout this range, and calculations of the yield of sulfatide added to water vs urine were similar, indicating that there is little or no matrix effect.

Determinations of precision. The intraassay imprecision (CV) of the urinary sulfatide assay, determined by measuring the urinary sulfatide concentrations in four aliquots of a normal control urine, was 27%. For interassay precision the urinary sulfatide concentrations were measured on 10 different occasions for one disease (MLD) control urine and on 5 different occasions for a normal control urine; the respective inter assay CVs were 30% and 23%.

Determination of yield. The yield of the sulfatide standard, 100 μg in 20 mL of distilled water, was calculated with each run. Seventeen such analyses gave a mean recovery of 29% ± 11%.

EVALUATION OF URINARY SEDIMENT

Philippart et al. suggested that the majority of urinary sulfatide is in the urinary sediment [14]. Consequently, some methods of urinary sulfatide analysis collect and analyze the urine sediment, discarding the nonparticulate fraction [14, 18–20]. We centrifuged urine specimens from two individuals with MLD to determine whether the sulfatide was distributed principally in the pellet (i.e., the particulate fraction) or the supernate; 64% and 67% of the sulfatide was present in the particulate fraction in these two cases (Table 1). Therefore, it is important to thoroughly mix each urine sample before taking an aliquot for analysis.

Fig. 3. Linearity of sulfatide analysis and limit of detection in water matrix (left) or in a normal control urine (right).
(Left) Sulfatide standard (50–6400 pmol) was added to 20 mL of distilled H_2O and analyzed in triplicate. Linear regression analysis of the curve gave y = 0.05x – 1.029 (r^2=0.944). (Right) Sulfatide standard (1000–11 000 pmol) was added to 20-mL aliquots of a normal control urine and analyzed in triplicate. Linear regression analysis of the curve gave y = 0.021x + 0.059 (r^2=0.91);
CLINICAL STUDIES

Urine specimens from 18 healthy controls and 20 individuals having clinically and enzymatically confirmed MLD were analyzed for their sulfatide content. The separation of values between the normal controls [mean ± SD 0.16 ± 0.07 nmol/mg creatinine (range: 0.07–0.34)] and the individuals with MLD [7.6 ± 6.1 nmol/mg creatinine (range: 1.2–24.2)] was excellent (Fig. 4). Individuals having genetic lysosomal storage disorders other than MLD did not have increased urinary sulfatide concentrations (data not shown).

Discussion

Several methods for the analysis of urinary sulfatides have been developed and applied to the analysis of sulfatiduria in individuals having MLD. The method utilized by Philippart et al. involved purification of urinary sulfatides by sequential use of DEAE cellulose and silicic acid chromatography, followed by two-dimensional thin-layer chromatography and gas-liquid chromatography [14]. That study provided confirmation of earlier qualitative analyses [13] showing marked differences in the urinary sulfatide concentrations between normal individuals and persons with MLD. The method used was impractical for routine clinical diagnostic purposes, for it required large quantities of urine (24-h urine collections from affected individuals and 10–30 L from controls) and large SDs were noted for each group [14]. In contrast, the assay described here requires only 10–20 mL of urine and has good precision.

Nonaka and Kishimoto developed an efficient method for isolating and quantifying sulfatides from tissues by using per-benzoylation and HPLC separation [23]. However, their method was limited by high background and suboptimal resolution of sulfatides from other lipids [23]. Our method provides clear separation of sulfatides from other urinary lipids (Fig. 2).

Brown et al. used the method of Nonaka and Kishimoto to analyze the urinary sulfatide concentrations of three patients with MLD. They found sulfatide concentration of 1.8 to 4.8 nmol/mg creatinine in those patients, whereas the control had a value of 0.02 nmol/mg creatinine [17], consistent with our results. Shimomura and Kishimoto improved on that method by using a single-phase system for the desulfation reaction, which was simpler and less technically demanding than the two-phase system in their earlier method [24]. In addition, the newer method had better sensitivity and resolution of the sulfatides from other lipids. Nonetheless, under the HPLC chromatographic conditions they used, multiple uncharacterized lipid classes coeluted with the sulfatides [24]. Strasberg et al. [18], using a method similar to that of Shimomura and Kishimoto but incorporating seminolipid as an internal standard, confirmed the earlier reports of marked sulfatiduria in MLD urines. Their method was limited by suboptimal sensitivity, suboptimal resolution from other lipids, and the small number of MLD specimens they analyzed. Molzer et al. [20] and Uyama et al. [25] reported thin-layer chromatography-based assays of urinary sulfatides; few details regarding the resolution and other aspects of the performance of the assays were provided by either group.

All of the methods for urinary sulfatide analysis described above have been applied to very limited numbers of samples, and some of them are impractical for routine clinical use. In addition, most of the methods were not rigorously evaluated with respect to many of the standard clinical chemistry characteristics, e.g., intra- and interassay precision analyses, or the determination of adequate reference intervals. In some instances, no information regarding the method of sulfatide analysis was provided [15, 16].

No one, including ourselves, has reported on the intraindividual variation of urinary sulfatide concentrations in persons with MLD. Such variation might account, at least in part, for the large range of urinary sulfatide concentrations we noted in our population of individuals with MLD. Such a study has presumably not been done because very few laboratories perform this test and because a repeat urinary sulfatide determination is unnecessary for individuals who have had a previous positive analysis that led to or confirmed a diagnosis of MLD. Insofar as bone marrow transplantation is currently offered as an experimental therapeutic approach for enzyme replacement therapy in
selected individuals with MLD, and because urinary sulfatide may be a useful analyte for following pre- and postbone marrow transplantation, we plan to assess the intraindividual biological variation of this analyte.

The method described here has excellent sensitivity and is linear over an extensive and clinically relevant concentration range. The assay has good precision, and the recovery of sulfatide from urine is excellent in view of the ~30% yield of sulfatide standard. In addition, the sulfatide-derived galactosylceramides are well resolved from other lipids. Finally, the separation between the urinary sulfatide concentrations of individuals with MLD and those of normal controls is also excellent. Consequently, the method can be used to evaluate clinically atypical cases in which a diagnosis of MLD is a clinical consideration. It also can be used to evaluate enzymatically atypical cases when the determination of the leukocyte and (or) fibroblast ASA activity cannot unambiguously establish the diagnosis. The method should also be valuable in the diagnosis of activator deficiency forms of MLD because of the associated sulfatiduria, as well as the diagnosis of multiple sulfatase deficiency disease. Those two conditions are extremely rare; we have seen no specimens from the former but have noted pathologically increased sulfatiduria in a case of multiple sulfatase deficiency, and others have noted increased urinary sulfatides in several cases of those conditions as well [1].

The method described here should also be valuable in the evaluation of individuals who are homozygotes for the common ASA pseudodeficiency allele or who are heterozygotes for that allele and an MLD allele. Each of these two genotypes is currently believed to correspond to a benign phenotype [26, 27], although there has been concern that the compound heterozygote state may either be associated with or predispose to dysfunction in the central nervous system [28]. Nine pseudodeficient/MLD compound heterozygotes studied recently by Penzien et al. [27] did not have markedly increased urinary sulfatide concentrations, which, together with additional clinical data from those persons, argues that individuals with that genotype will not have severe forms of MLD and may have few or no manifestations from their low ASA activity. We are also engaged in a prospective analysis of individuals with this genotype, and our preliminary data suggest that individuals who are compound heterozygotes for MLD and pseudodeficiency genes have mildly to moderately increased urinary sulfatide concentrations, in contrast to the finding of Penzien et al. [27]. The definitive resolution of this issue is of considerable importance in view of the common occurrence of the pseudodeficiency alleles in both healthy and symptomatic populations. The utilization of the assay described here promises to be helpful in clarification of the numerous instances of clinically or enzymatically complex situations in which a form of MLD is a concern.

References


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