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Direct tandem mass spectrometric profiling of sulfatides in dry urinary samples for screening of metachromatic leukodystrophy

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ABSTRACT

Background: Prediagnostic steps in suspected metachromatic leukodystrophy (MLD) rely on clinical chemical methods other than enzyme assays. We report a new diagnostic method which evaluates changes in the spectrum of molecular types of sulfatides (3-O-sulfogalactosyl ceramides) in MLD urine.
Methods: The procedure allows isolation of urinary sulfatides by solid-phase extraction on DEAE-cellulose membranes, transportation of a dry membrane followed by elution and tandem mass spectrometry (MS/MS) analysis in the clinical laboratory. Major sulfatide isoforms are normalized to the least variable component of the spectrum, which is the indigenous C18:0 isoform. This procedure does not require the use of specific internal standards and minimizes errors caused by sample preparation and measurement.
Results: Urinary sulfatides were analyzed in a set of 21 samples from patients affected by sulfatidosis. The combined abundance of the five most elevated isoforms, C22:0, C22:0-OH, C24:0, C24:1-OH, and C24:0-OH sulfatides, was found to give the greatest distinction between MLD-affected patients and a control group.
Conclusions: The method avoids transportation of liquid urine samples and generates stable membrane-bound sulfatide samples that can be stored at ambient temperature. MS/MS sulfatide profiling targeted on the most MLD-representative isoforms is simple with robust results and is suitable for screening.

1. Introduction

Metachromatic leukodystrophy (MLD) is a rare autosomal recessive disorder caused by a deficiency of lysosomal arylsulfatase A (ASA). ASA desulfates 3-O-sulfogalactosyl ceramide (sulfatide, galactosylceramide I^3-sulfate) (Fig. 1) and is assisted by the nonenzymatic activator protein saposin B [1,2]. ASA deficiency, or lack of its activator protein causing saposin B and prosaposin deficiencies (Psap-d), results in accumulation of sulfatides in lysosomes and demyelination of the central and peripheral nervous system. The disease has several variants that differ in the clinical onset and severity. The late infantile form of MLD is most common and severe and results in death of the affected children in the first decade of life. The juvenile and adult forms have a milder course that manifests itself by gait disturbances, mental regression, and emotional disturbances [2].

Biochemical detection of MLD presents specific challenges. Since there is currently no approved therapy for MLD [3–7], detection of the late infantile form by newborn screening has not been considered, and a suitable method is not available so far. Enzyme assays in cell homogenates have been developed for ASA that use 4-nitrocatechol sulfate for UV/VIS or methylumbelliferyl sulfate for fluorescence detection of products [8–11]. However, the practical use of enzyme assays is hampered by the occurrence of two pseudodeficiency alleles of the ASA gene locus (EC 3.1.6.8) which are carried by a substantial group of the general population, estimated at 10 − 15% in Europe [2]. These benign pseudodeficiencies are caused by either a point mutation that removes the protein glycosylation site [12], or a mutation of a polyadenylation site [13], resulting in low ASA activity in *in vitro* assays, but not causing disease [14–17]. Other methods of diagnosis are based on clinical symptoms and changes caused by demyelination and deposition of sulfatides in metachromatic granules [18,19].

All MLD forms manifest themselves by an elevated concentration of sulfatides in urine. Previous bioanalytical methods of sulfatide detection were based on thin layer chromatography separation of native glycosphingolipids with densitometric evaluation [20], or utilized chemical derivatization combined with gas [21] or liquid [22] chromatography, or applied matrix-assisted laser desorption ionization mass spectrometry after sulfatide conversion into its lysoform [23]. Recently, Meikle's group reported detection and quantitation

Abbreviations: CV, coefficient of variation; DEAE, Diethylaminoethyl; MLD, metachromatic leukodystrophy; Psap-d, prosaposin deficiency; ASA, arylsulfatase A; PTFE, polytetrafluoroethylene; SRM, selected reaction monitoring; MS/MS, tandem mass spectrometry; S/N, signal to noise ratio; IPN, isoform profile number (ratio of the sum of the major five isoforms and the C18:0 sulfatide); DUS, dry urinary sample.

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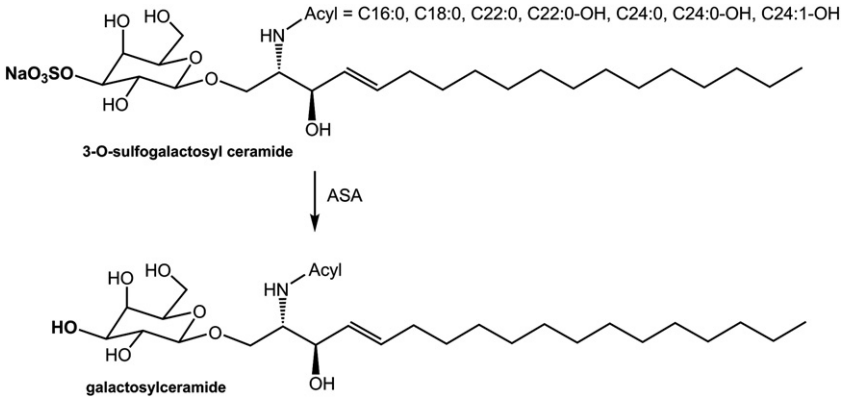

Fig. 1. The chemistry of sulfatide degradation catalyzed by arylsulphatase A.

of urine sulfatides by electrospray ionization mass spectrometry in the negative ion mode [24,25]. Norris et al. [26] have developed an assay for sulfatide detection in brain tissue that was based on positive ion electrospray tandem mass spectrometry of sulfatide–lithium ion adducts. Kuchař et al. [27] used tandem mass spectrometry in the negative ion mode to prove massive excretion of urinary sulfatides in patients with function defect of ASA protein activator saposin B (prosaposin and saposin B deficiencies) and also in MLD cases. A common feature of these procedures is that they use liquid–liquid extraction from urine to chloroform–methanol.

Tandem mass spectrometry provides specific data that allow examination of detailed patterns of molecular species and monitoring of changes in their relative abundance that can be related to the nature of the disease. Detailed MS/MS determination of variations in lipid composition has strongly influenced the clinical view in some diseases, and understanding such variations in relation to the disease may help identify new categories of biomarkers [28].

We now report a new laboratory procedure that uses ion-exchange membranes for an efficient and solvent-free sulfatide extraction from urine and produces dry samples that are readily transported and stored. Electrospray MS/MS is used for targeted lipidomic analysis of selected sulfatide isoforms for screening of MLD.

2. Materials and methods

2.1. Materials

Archived and anonymous urine samples (first morning or randomly taken specimens) were obtained from patients previously diagnosed with MLD (20 patients) or prosaposin deficiency (one patient), and controls (50 individuals). The study was approved by the Ethics Committee of the General University Hospital in Prague. MLD samples were obtained from patients afflicted with the late infantile form (7 patients, ages 2–5), juvenile form (4 patients, ages 7–17), and adult form (9 patients, ages 23–38). The prosaposin deficiency sample was from a 44-day old male infant. Samples (10 ml) were stored in plastic tubes at −20 °C. DEAE membranes were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany, Sartobind D membrane A4 cat No. 94IEXD42-001). Chloroform (Sigma-Aldrich Co., St. Louis, MO; grade CHROMASOLV for HPLC 99.9%), methanol (Sigma-Aldrich; grade CHROMASOLV for LC–MS Riedel-de Haën or Fluka), *n*-hexane (Sigma-Aldrich; grade CHROMASOLV for LC–MS Fluka), 2-propanol (Sigma-Aldrich; grade CHROMASOLV for LC–MS Fluka) and ammonium acetate (Sigma-Aldrich, Fluka Analytical, puriss p.a. for mass spectroscopy ≥99.0%) were used as received. SUPELCOSIL ™ LC-Si HPLC Column 7.5 cm × 3 mm, 3 μm, was purchased from Supelco (Cat No 58980C30, Supelco, Bellefonte, PA, USA). Security Guard Kit (Phenomenex, KJO-4282) and Silica Cartridges (10 pcs, Phenomenex, AJO-4348) were supplied by Phenomenex Inc. (Torrance, CA, USA). C12:0 sulfatide was purchased from Avanti Polar Lipids Inc (Alabaster, AL, USA). C17:0 sulfatide was prepared as described previously [29]

2.2. Preparation of dry DEAE membrane with bound urinary sulfatides

DEAE membrane (Sartobind D, A4 29.7 × 21 cm) was cut into 1 × 1 cm squares. The squares were immersed for 15 min in the sufficient volume of urine (about 10 ml is recommended) which was previously thoroughly mixed for 1 min to disperse the sediment throughout the sample volume. The soaked DEAE membrane was placed on a laboratory stand and allowed to dry at laboratory temperature for about 5 h. No blotting material was used to speed up drying. The dried DEAE membranes (DUS) were stored at −20 °C prior to further processing.

2.3. Extraction of sulfatides from DEAE membrane

Sulfatides were extracted by ion-exchange using 0.2 M ammonium acetate in methanol. A membrane square was placed in a 1.5 ml Eppendorf tube with 1.4 ml 0.2 M ammonium acetate in methanol. This was followed by 30 min vortexing at 1400 rpm at room temperature. After intensive vortex mixing, the methanol solution was transferred to another Eppendorf tube and the solvent volume was reduced to <50 μl by evaporating under a stream of nitrogen at 40 °C for up to 20 min. Then 300 μl of MilliQ water was added followed by 700 μl of chloroform:methanol (2:1, v/v) and the mixture was vortexed at 1400 rpm for 30 min. The organic and water phases were separated by centrifugation at 14 000 × *g* for 5 min. A 340 μl portion of the organic phase was transferred to a glass vial and dried under stream of nitrogen. The residue was dissolved in 500 μl of methanol prior to tandem mass spectrometry analysis.

2.4. Tandem mass spectrometry

Mass spectra were measured on an ABI/MDS SCIEX API 3200 tandem mass spectrometer equipped with an ESI source and coupled to an Agilent HPLC 1100 series. Samples (20 μl of methanol solution) were introduced by flow injection at a mobile phase flow rate of 50 μl/min and electrosprayed in the negative ion mode to form $[M–H]^-$ ions. Generated ions were analyzed by SRM of precursor ions (Supplementary data Table S1) and the common HSO_4^- (*m/z* 97) fragment ion. Detailed instrument settings are described in the Supplementary data section.