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Direct Tandem Mass Spectrometric Profiling of Sulfatides in Dry Urinary Samples for Screening of Metachromatic Leukodystrophy

Ladislav Kuchař, **Befekadu Asfaw**, **Helena Poupětová**, **Jitka Honzíková**[+], **František Tureček**[*], and **Jana Ledvinová**[*]

Institute of Inherited Metabolic Diseases, First Faculty of Medicine and General Teaching Hospital, Charles University, 128 08 Prague, Czech Republic

[+]Department of Chemistry, University of Washington, Seattle, WA 98195-1700, USA

Abstract

Background—Prediagnostic steps in suspected metachromatic leukodystrophy (MLD) rely onclinical 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.

Keywords

urinary sulfatide; isoforms; screening for metachromatic leukodystrophy; tandem mass spectrometry; DEAE-cellulose membrane; dry urinary samples

[*]Corresponding author: Jana Ledvinová, Institute of Inherited Metabolic Diseases, First Faculty of Medicine and General Teaching Hospital, Charles University, 128 08 Prague, Czech Republic; Tel: +420 224967034, Fax.: +420 224967119, jledvin@cesnet.cz: František Tureček, Department of Chemistry, University of Washington, Seattle, WA 98195-1700, USA, Phone +1 206-685-2041, Fax +1 206-685-8665, turecek@chem.washington.edu.

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 lyso-form [23]. Recently, Meikle's group reported detection and quantitation 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-days 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 Table S1) and the common

HSO_4^-(*m/z* 97) fragment ion. Detailed instrument settings are described in the Supplementary Data section.

2.5. Evaluation of Sulfatide Isoform Profile: Calculation of Isoform Profile Number

The measured SRM peak heights of major MLD-related isoforms (C22:0, C22:0-OH, C24:0, C24:1-OH, and C24:0-OH see Fig. 2) were recorded and normalized to that of the indigenous C18:0 isoform to obtain the isoform profile number (IPN). Thus, the IPN value represents the ratio of the summed signals of five major elevated isoforms to the signal of the least variable C18:0 isoform.

The peak heights are proportional to the sulfatide concentrations through the corresponding response factors depending on the ionization efficiency, ion transfer, dissociation efficiency of $[M–H]^-$ ions, and fragment branching ratios. The data thus also reflect the relative abundance of each isoform in urine and are used for profiling samples from affected patients and controls.

2.6. Determination of analytical precision of the DEAE membrane method, linearity, and limits of quantification

Coefficients of variation (CV) of IPN were calculated to determine the precision of inter- and intra- assay measurements. For intra-assay analysis, one control and one MLD urine sample were used for preparation of DEAE membrane bound sulfatides. Sulfatides were extracted from the dry membrane as described above and the extracts were repeatedly analyzed by MS. The intra CV was calculated from 10 measurements.

For inter-assay analysis, series of 10 samples of DEAE bound sulfatides were prepared from one control and one MLD urine sample. The Inter CV was determined after analysis of 10 control and 10 MLD sulfatide extracts.

To determine the concentration dependence of the analytical precision, we used a standard sample of bovine brain sulfatides dissolved in methanol and applied on DEAE membranes. IPN was evaluated in DEAE membrane eluates over a broad range of concentrations (8 concentration points) varying from 10 to 5000 ng/membrane.

Limits of quantification for individual sulfatide isoforms in DUS were determined by a previously published method [27] using a C12:0 sulfatide internal standard.

To determine the linearity of the method, we used a standard sample of bovine brain sulfatides dissolved in methanol and applied to DEAE membranes. Signals of evaluated isoforms were measured over a broad range of concentrations from 10 to 20000 ng of sulfatides per membrane.

2.7. Evaluation of Matrix Effects

Matrix effects of the co-eluted urine components from the DEAE membrane on the sulfatide signal were assessed in two ways, e.g., using a post-extraction technique and a post-column infusion method. The latter provides the ability to design and modify methods to eliminate adverse effects of the matrix [30]. Briefly, an infusion pump is used that delivers a constant flow of the analyte or standard into the LC eluent before the ESI source which, allows one to evaluate the influence of matrix on the analyte signal during the LC run [30].

The post-extraction addition method is based on a comparison of the different signal responses of the analyte with added matrix and dissolved only in a pure solvent.

Ion suppression (matrix) effects were examined by the postextraction addition technique in six urine samples representing six independent sources of the same matrix (3 controls, 3 MLD) within a large range of creatinine concentrations (2.3-17.0 mmol/l) using published protocols [30-32]. DEAE membrane eluates were processed as described above, and the organic and water phases were separated. 340 μl of the organic (lower) phase was mixed with 10 μl of the C17:0 isoform (40 ng) solution, and the suppression of C17:0 sulfatide ion signal intensity was monitored for each urine sample.

We used post-column infusion analysis [30] with the C12: 0 sulfatide standard to test whether matrix effects can be eliminated or reduced by HPLC (silica column) or LC separation (Security Guard Kit with silica cartridges; see details in the Supplement).

A sample was prepared by mixing the abovedescribed six urine samples and used as a source of matrix components. Postcolumn infusion of C12:0 sulfatide standard (40ng in 500μl of 2-propanol:H2O; 90:10; v/v) at flow rate of 10 μl/min was used for matrix effect evaluation.

2.8. Stability of sulfatides on DEAE membrane

Sulfatides from six urine samples (3 controls and 3 MLD) were bound to the DEAE membrane as described and stored at laboratory temperature (l.t.) and at −20 °C prior to further processing. Samples were processed at different time intervals in the course of 100 days and individual IPN were calculated.

2.9. Kidney sulfatides analysis

For tissue lipid analysis, lipid extracts of a formaline-fixed control kidney and a kidney from the patient with prosaposin deficiency were prepared by successive extraction of tissue homogenates with chloroform:methanol:water mixtures as described [33, 34]. Aliquots corresponding to 1 mg of the wet weight of extracted tissue were analyzed by tandem mass spectrometry according to the previously published method [27].

3. Results

3.1. Analytical Precision of a DEAE Membrane Method, Linearity and Limits of Quantification

The average CV within the range from 10 to 5000 ng sulfatides/sample was 7%. The analytical precision of the method gave an intra CV = 11% from repetitive analysis ($n = 10$) of one control sample. The MLD sample gave an intra CV = 4% ($n = 10$).

The inter CV was calculated from analyses of 10 DEAE membrane eluates. The Inter CV was 13% and 11% for the MLD and control samples, respectively.

Limits of quantification were determined for each evaluated isoform in urinary samples where the signal to noise ratio was at least 10:1. Under these conditions, the limits of quantification were 4 pmol of one specific urinary sulfatide isoform per cm^2 of DEAE membrane.

Signal responses of individual isoforms were linear in a broad range of concentrations from 5 to 1500 pmol applied to the DEAE membrane, which is consistent with the concentration range for the determination of sulfatides in control and MLD urines.

3.2. Matrix Effects

Because the urine samples contain the sulfatides in the presence of a large excess of low molecular mass compounds (matrix), the determination of matrix effects on the extraction and mass spectrometric analysis was essential [30]. DUS extracts showed matrix effects resulting in C17:0 sulfatide ion signal suppression by 76-84% due to the presence of extracted co-ionized urine components. However, the ion signal intensity of analyzed sulfatide isoforms was sufficient for reliable analysis. The ESI ion intensities of other sulfatides, for which we did not have synthetic standards, are presumably suppressed to a similar extent.

Nevertheless, we have tried to suppress the matrix effect using two alternative methods, one using HPLC coupled to ESI-MS/MS and the other using Security Guard Kit with two Silica cartridges (LC), which could be a fast and simple alternative to the HPLC method (for details see the Supplement).

HPLC fully removed matrix effects (Supplementary Fig. 1A), which was confirmed by post-extraction addition and also by the post-column infusion method. LC utilizing a Security Guard Kit with two Silica cartridges reduced the signal intensity suppression from 75% to 17% (Supplementary Fig. 1B) which was also confirmed by the methods mentioned above. Despite these benefits, the direct FIA-MS/MS was both faster and easier to manage while providing a rapid and reliable examination of suspected cases, which we verified in 20 MLD patients and 50 controls.

3.3. Stability of sulfatides on DEAE membrane

The stability of urinary sulfatides that were bound on DEAE membrane and stored from 1 to 100 days at laboratory temperature (l.t.) and at −20°C was studied for six different urine samples with high and/or low levels of sulfatides (3 control and 3 MLD urines). The evaluation of the IPN measurements is summarized in Table 1. The IPN values were relatively stable in the course of 100 days of storage and provided a clear distinction between values for patients and controls.

3.4. Evaluation of MLD-related isoforms in the total spectrum of urinary sulfatides

Five sulfatide isoforms C22:0, C22:0-OH, C24:0, C24:1-OH, and C24:0-OH which were most distinctly elevated in sulfatidoses (major isoforms; Fig. 2), were selected as being representative for analysis. In sulfatidoses, these major isoforms collectively accounted for 64% of total sulfatides.

The least variable parameter of the total profile was the C18:0 isoform which was therefore used for normalization of the five major elevated isoforms. To evaluate its variability, the percentage of C18:0 isoform of total sulfatides was calculated for 26 individual control and 10 MLD samples. The percentage of the C18:0 isoform in the total sulfatide spectrum was 7.0 ± 2.0% for controls (n = 26, creatinine levels 2.3 - 12.2 mmol/l) and 2.0 ± 1.0% for MLD patients (n= 10, creatinine 3.3 - 17 mmol/l) and prosaposin deficient patient (creatinine 0.4 mmol/l).

3.5. Isoform Profile Number (IPN) - a new MLD biomarker

The ratio of the combined signals of the *major isoforms* relative to the intrinsic C18:0 sulfatide reference (IPN) provides a clear-cut diagnostic parameter as shown in Fig. 3 for the set of 50 control samples, 20 MLD affected patients (all three clinical variants), one MLD heterozygote and one prosaposin deficient patient.

3.6. Comparison of kidney and urinary sulfatide isoform profiles

The comparison of kidney and urinary sulfatide molecular types under normal conditions and in case of prosaposin deficiency is shown in Fig.4. The data indicate that the urinary and kidney profiles from the control samples are distinctly different (Fig.4A). The urinary profile from the prosaposin deficient patient differs from those of the controls (see Fig.2) and follows the profile in the kidney (Fig.4B).

Both the patient's and control kidney patterns show a similar composition of the major isoforms regardless of the absolute values for total sulfatides (85μg/g tissue wet weight for control versus 3000μg/g for the prosaposin deficient kidney).

4. Discussion

We have shown that a simple profiling of specifically increased sulfatide isoforms reliably identified all MLD and prosaposin deficient patients, indicating no false negatives, and distinguished all controls, indicating no false positives (Fig. 3). The implementation of DEAE membranes facilitates sample transportation, handling of large sample sets, and enables the partial purification of urinary sulfatides and thus higher sensitivity of the measurements. These are the key characteristics of the procedure that could be introduced as a screening method for MLD because enzyme-based dry-blood-spot analysis is still missing [35].

Selection of five MLD-related isoforms minimizes individual differences in collected samples in comparison with the previously reported analysis of Gb3Cer profile in urine of Fabry patients based on only one most elevated C24:0 isoform [36].

In an effort to simplify the analytical procedure and lower the cost, we sought the least variable component of the sulfatide spectrum to normalize the signal of major elevated isoforms. We found the sulfatide C18:0 isoform, which was the most stable parameter percent wise, and used it as an indigenous reference for calculating the isoform profile number IPN as a disease marker.

The low variability of the C18:0 isoform in the profile is possibly due to its constant production in biosynthetic pathways where the corresponding synthases preferentially synthesize the isoforms with long acyl chains C20-C24, thus keeping the amount of C18:0 isoform less variable [37, 38].

Utilizing the C18:0 isoform as a normalizing parameter avoids isotope or mass-labeled external standards that are commonly used in other methods [24, 25, 27]. In addition, the C18:0 reference isoform is naturally present in the analyzed material along with the measured isoforms and thus compensates for errors caused by sample processing, e.g., affinity binding, elution and extraction, and also for matrix effects [39].

The use of the C18:0 reference isoform also helps to resolve the long-standing problem of suitable evaluation of urinary lipids that results from inappropriate normalizing parameters (e.g. creatinine) [40, 41]. Concentration of urine does not affect the measurement and evaluation using IPN values. Both types, the first morning specimens or samples taken randomly during the day may be used, but always perfectly mixed before any further handling.

The described method was thoroughly characterized by determining the analytical parameters and DUS storage stability. CV of IPN values did not depend on the total sulfatide level across a broad range of sample loadings which is essential for the method reliability. Intra- and inter- assay CV were always below 13%. This indicates that the *IPN*

values are not affected by selective binding of some sulfatide isoforms to the DEAE membrane.

It should be noted that it is essential to achieve adequate signal-to-noise ratios (S/N) of the measured data to evaluate correct IPN. The recommended minimum for the "quantitative" data is S/N ≥ 10 [31]. Our DEAE membrane method utilizing FIA-ESI-MS/MS revealed S/N ratios about 25 for control samples and 47 for MLD. This demonstrates that direct FIA-ESI-MS/MS provides reliable results. LC separation is not necessary to increase the S/N ratio, but represents an alternative method. Examples of measured data are shown in Supplementary Fig. 2.

The stability of sulfatides on DEAE membrane under different storage conditions was found sufficient, as the IPN value provided clear differentiation between the control group and MLD patients at any time point up to 100 days of storage (Table 1). Samples are usually delivered to the analytical laboratory within one week after collection but even longer storage period does not have any negative impact on the outcome of the laboratory analysis and diagnosis.

Another advantage of the method is its ability to diagnose saposin B deficiency caused by mutations of prosaposin encoding gene. This rare disease is easily detectable by determination of sulfatide IPN in urine, although the *in vitro* enzyme assays fail.

Despite the differences among individual patients, the concentrations of the most abundant isoforms correlated with the total sulfatide values in all examined samples (Supplementary Fig. 3). We assume that the method may also have the potential to distinguish common ASA pseudodeficiencies (ASA PD) which show low *in vitro* ASA activities but do not result in morbidity in ASA PD individuals who do not present significantly elevated urinary sulfatide levels [2, 25]. This issue will be further investigated in dependence on the availability of PD ASA material.

The cause for the elevated urinary C22:0, C:22:0-OH, C24:0, C24:1-OH, and C24:0-OH sulfatide isoforms has not been exactly determined so far. It is presumed that the exfoliated renal tubule epithelium cells affected by lysosomal storage are the primary source of excreted sphingolipids in some LSD [2, 42-44]. In the absence of renal damage, the renal epithelium cells appear in the urinary sediment in only very small quantities [45, 46].

We compared the sulfatide profiles in urine and in extracts of kidney homogenates from normal individuals and from the prosaposin deficient patient [41].

The pattern of sulfatide isoforms was similar in the healthy kidney and in the kidney affected by lysosomal storage (Fig. 4 A,B; purple highlighted bars).

In contrast, an altered urinary sulfatide profile (Fig. 4, blue bars) was found that exactly corresponded to the sulfatide kidney profile of the prosaposin deficient patient. This can be taken as an indirect evidence for changes in the composition of cellular types in the urinary sediment - desquamated urothelial cells in controls [45] and lipid-laden renal tubule cells [43] in patients with LSD.

The spectrum of the sulfatide isoforms in the kidney to a large extent corresponds to the expression of ceramide synthases (CerS, acyl-CoA:sphinganine N-acyltransferase, E. C. 2.3.1.24) that catalyze acylation of sphinganine in the endoplasmic reticulum [47]. The CerS2 form, which is mainly expressed in kidney cells, is specific for acylation of sphingoid base with C22 through C24 fatty acids [38, 47] which are major molecular forms of kidney

sulfatides and are elevated in urine of patients with MLD and prosaposin deficiency. Expression of CerS in urothel has not been investigated so far.

5. Conclusion

DEAE-bound urinary sulfatide profiling by tandem mass spectrometry offers a robust method for laboratory diagnosis of patients affected with MLD and prosaposin deficiency. The method relies on the increase of specific molecular types of sulfatides in urine. The selection of only five major elevated isoforms and the C18:0 isoform as an indigenous normalizing parameter both simplifies the analysis and lowers the cost, as it does not require specifically labeled lipid standards and analysis of additional standardizing parameter such as creatinine, sfingomyelin etc. Incorporating the DEAE membrane to selectively capture the sulfatides from urine allows the preparation of dry samples with high stability of bound sulfatides which is easy for transportation and further analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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
MLD	metachromatic leukodystrophy

Highlights

- easy transportation of urine samples dried on DEAE-cellulose membranes
- partial purification of sulfatides
- reliable monitoring of specific changes in the sulfatide isoforms in MLD patients
- use of the indigenous sulfatide isoform (with C18:0 fatty acid) as reference
- simplified analytical procedure and lower cost of analysis important for screening

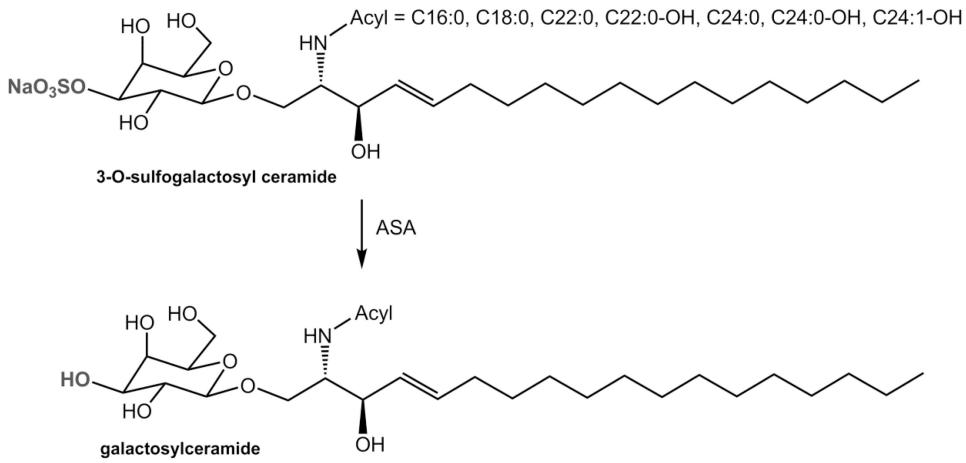

Fig. 1.
The chemistry of sulfatide degradation catalyzed by arylsulphatase A.

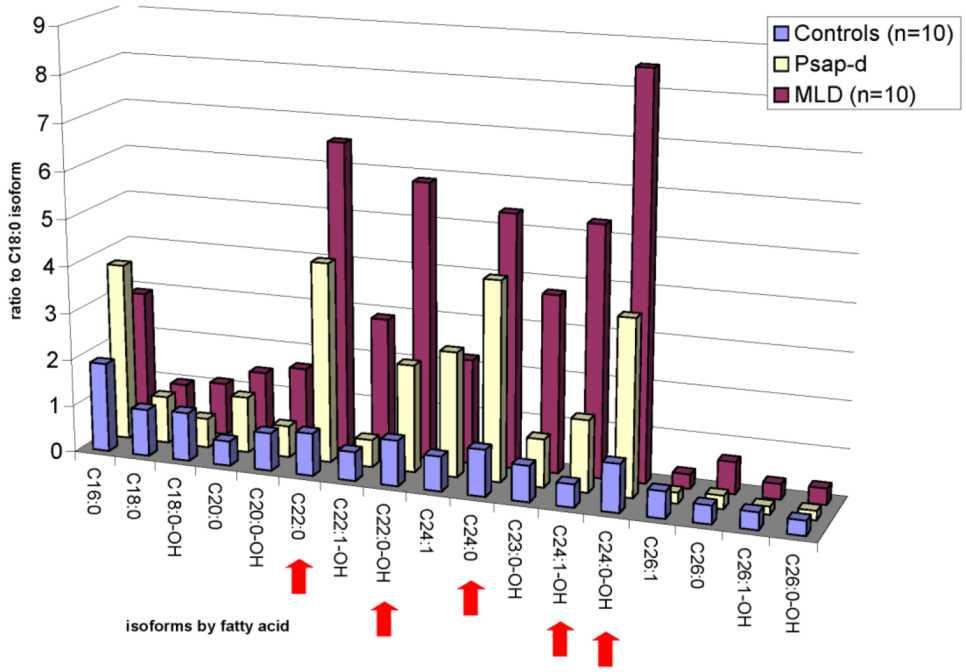

Fig. 2.

Comparison of urinary isoform profiles from MLD and prosaposin deficiency (Psap-d) patients and controls. Arrows indicate components undergoing major changes.

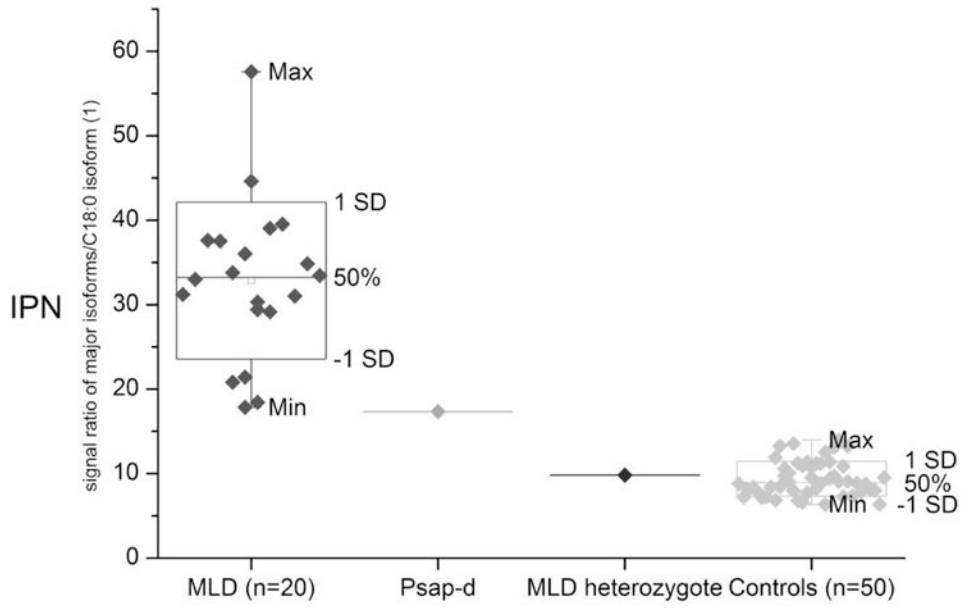

Fig. 3.

Ratio of composed SRM intensities of major urinary isoforms to the SRM intensity of the intrinsic C18:0 reference (IPN) for MLD and prosaposin deficiency (Psap-d) patients, a heterozygote, and controls.

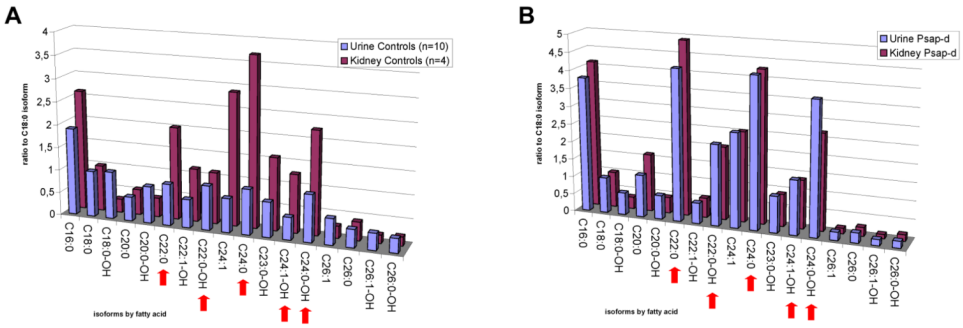

Fig. 4.

Comparison of sulfatide isoform urinary and kidney profiles in controls (panel A) and a prosaposin deficiency (Psap-d) patient (panel B). The similarity of urinary and kidney profiles in Psap-d is obvious. Arrows indicate major changes in the isoform profiles.

Table 1

Stability of sulfatides bound to DEAE membrane under different storage conditions evaluated by IPN

	Day						
	1	**3**	**6**	**100**	**Avg**	**SD**	**CV%**
C1 LT	7.69	6.73	8.06	6.07	7.14	0.91	12.71
FR	7.30	8.28	8.03	6.40	7.50	0.84	11.24
C2 LT	9.51	10.05	8.44	7.78	8.95	1.03	11.46
FR	8.50	8.34	9.55	8.80	8.80	0.54	6.12
C3 LT	8.62	9.86	10.26	6.79	8.88	1.56	17.57
FR	11.04	8.62	9.98	8.55	9.55	1.19	12.50
MLD1 LT	48.86	51.69	40.40	43.16	46.03	5.16	11.22
FR	36.42	37.19	48.27	35.53	39.35	5.98	15.20
MLD2 LT	32.33	20.63	25.30	20.50	24.69	5.56	22.53
FR	31.22	28.20	30.62	34.63	31.17	2.65	8.51
MLD3 LT	27.07	29.83	20.99	27.70	26.40	3.79	14.37
FR	21.76	24.20	24.32	24.99	23.82	1.42	5.95

IPN values are average of two measurements

C - Control; MLD - metachromatic leukodystrophy; LT - laboratory temperature; FR- storage in a freezer at −20 °C; values of IPN are in arbitrary units;