Clinica Chimica Acta 433 (2014) 39–43

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

# Quantification of sulfatides in dried blood and urine spots from metachromatic leukodystrophy patients by liquid chromatography/electrospray tandem mass spectrometry

Mariana Barcenas [a], Teryn R. Suhr [b], C. Ronald Scott [c], Frantisek Turecek [a], Michael H. Gelb [a,d,*]

[a] Departments of Chemistry, University of Washington, Seattle, WA 98195, United States
[b] MLD Foundation, 21345 Miles Dr., West Linn, OR 97068, United States
[c] Departments of Pediatrics, University of Washington, Seattle WA 98195, United States
[d] Departments of Biochemistry, University of Washington, Seattle, WA 98195, United States

ARTICLE INFO

*Article history:*
Received 11 November 2013
Received in revised form 8 December 2013
Accepted 12 December 2013
Available online 23 December 2013

*Keywords:*
Metachromatic leukodystrophy
Tandem mass spectrometry
Newborn screening
Dried blood spots
Lysosomal storage diseases
Urine
Sulfatides

ABSTRACT

*Background:* Treatments are being developed for metachromatic leukodystrophy (MLD), suggesting the need for eventual newborn screening. Previous studies have shown that sulfatide molecular species are increased in the urine of MLD patients compared to samples from non-MLD individuals, but there is no data using dried blood spots (DBS), the most common sample available for newborn screening laboratories.
*Methods:* We used ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) to quantify sulfatides in DBS and dried urine spots from 14 MLD patients and 50 non-MLD individuals.
*Results:* Several sulfatide molecular species were increased in dried urine samples from all MLD samples compared to non-MLD samples. Sulfatides, especially low molecular species, were increased in DBS from MLD patients, but the sulfatide levels were relatively low. There was good separation in sulfatide levels between MLD and non-MLD samples when dried urine spots were used, but not with DBS, because DBS from non-MLD individuals have measurable levels of sulfatides.
*Conclusion:* Sulfatide accumulation studies in urine, but not in DBS, emerges as the method of choice if newborn screening is to be proposed for MLD.



## 1. Introduction

Metachromatic Leukodystrophy (MLD) is a recessively inherited lysosomal storage disorder caused by the deficiency of the enzyme arylsulfatase A (ASA) resulting in the accumulation of 3-*O*-sulfogalactosyl ceramides (sulfatides) in tissues. The prevalence is 1:40,000 to 1:100,000 births [1,2]. Interest in newborn screening for MLD is increasing because of the recent development of potential therapies [3]. Although direct assay of lysosomal enzymes in dried blood spots is a powerful method for newborn screening of lysosomal storage diseases [4,5], application of this approach to MLD is problematic because trace amounts of ASA activity are sufficient to minimize disease severity and ASA pseudodeficiency is common in the population [6]. Healthy individuals with ~5% residual ASA activity are prevalent in the population [6], and even ~1–2% ASA activity is sufficient to lead to a non-MLD phenotype (unpublished data from A. Fluharty). Thus, it will be extremely difficult to develop a newborn screening of ASA enzymatic activity or protein abundance [7] that will not suffer from a high rate of false positives. It has been reported based on mass spectrometry results that the sulfatide substrates for ASA accumulate in urine of MLD patients [8–12]. However, almost all newborn screening laboratories only collect dried blood spots (DBS).

## 2. Materials and methods

### 2.1. Materials

C17:0-Sulfatide (sulfatide with a heptadecanoyl acyl chain on the amino group of the sphingosine backbone) and C24:0-sulfatide were obtained from Avanti Polar Lipids. These were also synthesized by treating psychosine with the appropriate fatty acyl N-hydroxysuccinimidyl ester followed by sulfation of the galactosyl unit using dibutyltin oxide and trimethylamine-$SO_3$ complex [13] using published procedures. Sulfatide species are abbreviated with the name of the fatty acid attached to the amino group of the sphingosine group, i.e. 24:1 is a 24-carbon fatty acid with 1 double bond, and 24:1-OH has a hydroxy group in the fatty acid chain.

Dried urine and blood samples were obtained with the help of the MLD Foundation. Institutional Review Board approval was obtained from the Univ. of Washington IRB panel. DBS were collected by puncturing the fingertip with lancet and letting the blood drip onto filter paper, which was air dried for ~2 h and then mailed over a few days to the Univ. of Washington. DBS from random, anonymous newborns were

* Corresponding author. Tel.: +1 206 543 7142; fax: +1 206 685 8665.
*E-mail address:* gelb@chem.washington.edu (M.H. Gelb).

0009-8981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2013.12.016

obtained from the Washington State Newborn Screening Lab after being stored at 18 °C for 8–10 months. Urine was collected on Whatmann 5 paper disks (70 mm), which were allowed to air dry for ~2 h at room temperature, then placed in a zip-lock plastic bag for shipment at ambient temperature. After arrival, all urine and DBS samples were stored at −10 to −20 °C in a closed jar with dessicant.

### 2.2. Extraction of sulfatides from dried urine and blood spots

To determine the extraction yield of sulfatides from the filter paper, a 3 mm paper punch (urine or blood) was spiked with 100 pmole of C17:0- and C24:0-sulfatide (from a stock solution in methanol (Fisher Scientific)), and the paper was allowed to dry for ~2 h. The punch was placed in a 1.5 ml polypropylene microfuge tube, and 1 ml of ethyl acetate (Macron Fine Chemicals) was added. The sample was mixed on a vortex mixer for 5 min and centrifuged at 3000 rpm for 3 min. Supernatant (0.8 ml) was transferred to another tube, and solvent was removed with a stream of nitrogen at room temperature. The residue was dissolved in 0.1 ml of methanol for injection into the mass spectrometer.

### 2.3. Processing of DBS and urine spots for sulfatide analysis

Samples for sulfatide analysis were processed as follows. A 10 mm punch of the DBS or urine spot was placed in a glass tube, and 1 ml of ethyl acetate was used for extraction as described above. The ethyl acetate added to each sample contained 2 pmole of C17:0-sulfatide as internal standard.

### 2.4. UHPLC/MS/MS analysis of sulfatides in DBS and urine spots

UHPLC was carried out with a Waters AQUITY system with a HSS T3 C18 analytical column (50 × 2.1 mm, 1.8 μm) with a HSS T3 (5 × 2.1 mm, 1.8 μm) VanGuard guard column (Waters Corp.). The elution solvent was water with 0.1% formic acid (solvent A) and 2-propanol/methanol (80/20) with 0.1% formic acid (solvent B) (all solvents are LC-MS Optima grade from Fisher Scientific). The solvent program was 82% solvent B to 92% solvent B over 1.3 min, hold at 92% solvent B for 1.7 min, all at a flow rate of 0.4 ml/min. The total run time was 3 min. Tandem mass spectrometry was carried out on a Waters Xevo TQ instrument used in negative ion mode. The injection volume was 10 μl. Mass spectrometer settings are given in Supplemental Material Tables 1 and 2.

## 3. Results

### 3.1. Extraction of sulfatides from dried blood and urine spots

We explored a number of organic solvents for extraction of sulfatides from filter paper ($CHCl_3$/methanol (2/1), hexane/2-propanol (3/2) and ethyl acetate) and found extraction yields for sulfatide of 69, 37 and 73%, respectively, thus ethyl acetate was used. When a methanol solution of C17:0- and C24:0-sulfatides was spotted onto filter paper followed by solvent removal, 74% and 76% of the sulfatide, respectively, were recovered after ethyl acetate extraction and subsequent quantification by UHPLC/MS/MS. Next we spotted known amounts of C17:0-sulfatide onto 1 cm diameter DBS or dried urine spots, and measured the recovery by UHPLC/MS/MS following extraction with ethyl acetate

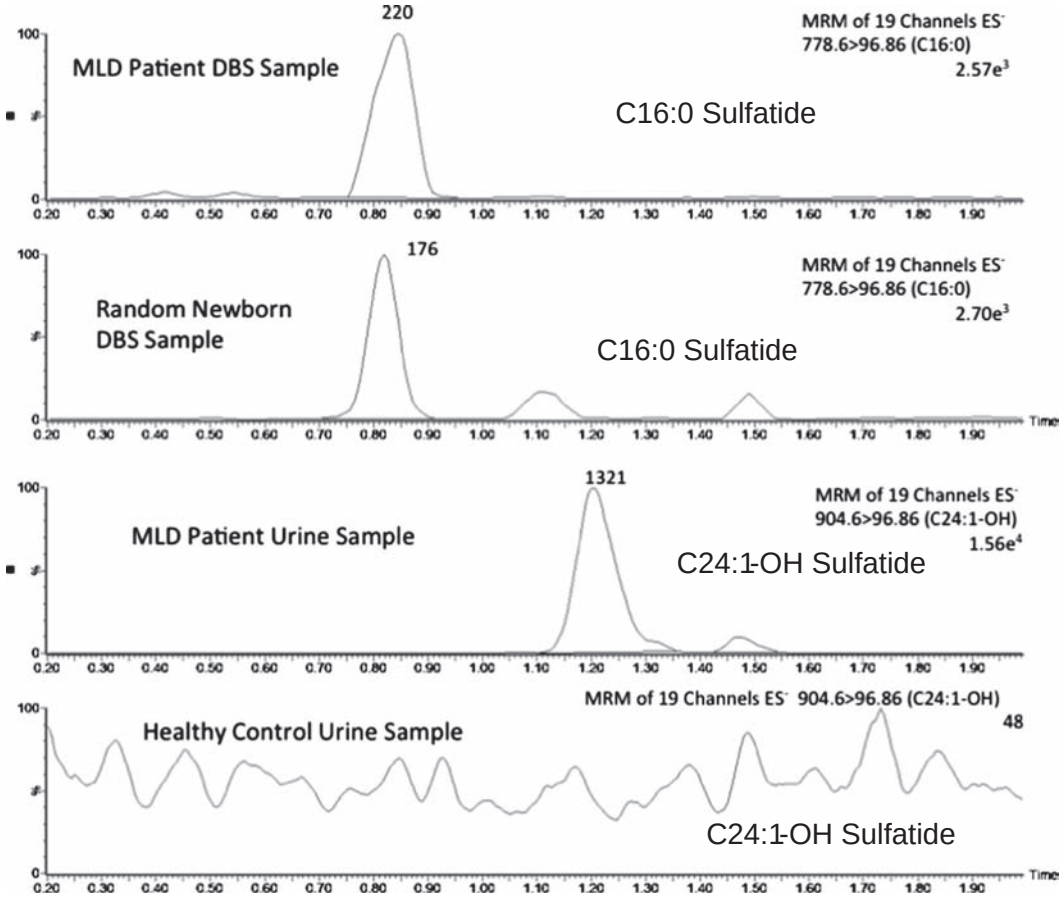


**Fig. 1.** Selective ion chromatograms of the indicated sulfatide molecular species in DBS from an MLD patient (top), in DBS from a non-MLD individual (second panel), in urine from an MLD patient (third panel), and in urine from a non-MLD individual (bottom panel). The Y-axis is Relative Intensity. The C17:0-sulfatide internal standard elutes at 0.85 min and gives a typical peak area of 3840 with a percent standard deviation of 17%.