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The stability of hexacosanoyl lysophosphatidylcholine in dried-blood spot quality control materials for X-linked adrenoleukodystrophy newborn screening

Christopher A. Haynes[*] and **Víctor R. De Jesús**

Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention, Atlanta, GA 30341, USA

Abstract

Objectives—Newborn screening for X-linked adrenoleukodystrophy utilizes tandem mass spectrometry to analyze dried-blood spot specimens. Quality control materials (dried-blood spots enriched with hexacosanoyl lysophosphatidylcholine) were prepared and stored at different temperatures for up to 518 days to evaluate the stability of this biomarker for X-linked adrenoleukodystrophy.

Design and methods—Dried-blood spot storage included desiccant (45, 171, and 518 days) or omitted desiccant (53 days at >90% relative humidity). Specimens were stored for 171 and 518 days at −20 °C, 4 °C, ambient temperature, and 37 °C. Each weekday for 45 days, a bag of specimens stored at 4 °C was warmed to ambient temperature and one specimen was removed for storage at −80 °C. Specimens were analyzed by high-performance liquid-chromatography electrospray ionization tandem mass spectrometry and data was plotted as concentration (micromoles per liter) vs. time. Linear regression provided slope and y-intercept values for each storage condition.

Results—Small slope values (0.01 or less) and y-intercept values close to the enrichment indicated less than 11% loss of hexacosanoyl lysophosphatidylcholine under all storage conditions tested.

Conclusions—Quality control materials for X-linked adrenoleukodystrophy are stable for at least 1 year when stored with desiccant.

Keywords

Tandem mass spectrometry; Dried-blood spot; X-linked adrenoleukodystrophy; Newborn screening; Lysophosphatidylcholine

Introduction

X-linked adrenoleukodystrophy (X-ALD[1]) is a peroxisomal disorder [1] caused by mutations in the peroxisomal transmembrane ALD protein (ALDP, ABCD1) [2]. The biochemical defect associated with X-ALD is an accumulation of very long-chain fatty

[*]Corresponding author at: 4770 Buford Hwy. NE, Atlanta, GA 30341, USA. Fax: +1 770 488 7459. cph7@cdc.gov (C.A. Haynes).

acids, which has been shown to result in the accumulation of C26:0-lysophosphatidylcholine (C26:0-LPC) [3]. Some newborn screening programs are either already performing newborn screening for X-ALD (New York) or may do so in the near future (Connecticut). Dried-blood spots (DBS) were prepared with C26:0-LPC-enriched whole blood, and stored under different conditions for up to 18 months. C26:0-LPC was measured to determine its stability under different storage conditions to evaluate candidate QC materials for X-ALD newborn screening.

Materials and methods

Reagents

Hexacosanoyl lysophosphatidylcholine (C26:0-LPC) and $^{2}H_{4}$-hexacosanoyl lysophosphatidylcholine ($^{2}H_{4}$-C26:0-LPC) were from Avanti Polar Lipids (Alabaster, AL). Ammonium acetate, HPLC–MS grade methanol and HPLC–MS grade acetonitrile were from Fisher Scientific.

Dried-blood spots

Packed red cells and serum were obtained from anonymous donors (Tennessee Blood Services), the red cells were washed 3 times with saline, and serum was added to achieve a final hematocrit of 50 ± 1% [4]. The blood was frozen and thawed to lyse the red cells, filtered through cheesecloth to remove small clots, enriched with C26:0-LPC, and then spotted onto Whatman 903 filter paper (100 μL spots). The blood spots were dried overnight and stored with desiccant at −20 °C.

Dried-blood spot enrichment

DBS Quality Control (QC) materials were prepared by enriching lysed blood with C26:0-LPC to target concentrations of 0 μM (no enrichment), 1 μM, and 10 μM. The C26:0-LPC stock solution was 1 mg/mL in methanol. Stability study materials were enriched to a target concentration of 4 μM (except the high-humidity stability study samples which were enriched to a target concentration of 10 μM). Using HPLC–ESI-MS/MS (see the HPLC–ESI-MS/MS analysis section), C26:0-LPC was measured in the DBS to assess homogeneity and characterize their enrichment. The means and standard deviations of the different materials were determined by analysis on 20 different days [5].

Stability studies

DBS enriched with C26:0-LPC were labeled to indicate their storage temperature and the number of days of exposure to that temperature. All DBS were placed in mylar zip-closure bags with desiccant (except the high-humidity samples), and bags were stored at −20 °C, 4 °C, ambient room temperature, and 37 °C. The high-humidity samples were placed in an un-zipped mylar bag (no desiccant) in a closed container with water-saturated paper towels at ambient room temperature. For the 6-month and 18-month stability studies a DBS sample

1X-ALD (X-linked adrenoleukodystrophy), ALDP (adrenoleukodystrophy protein), C26:0-LPC (hexacosanoyl lysophosphatidylcholine), DBS (dried-blood spot), QC (quality control), HPLC–ESI-MS/MS (high-performance liquid chromatography electrospray ionization tandem mass spectrometry).

was removed from each mylar bag (temperature condition) every 2 weeks and then stored at −80 C. Based upon results from the 6-month study, the samples intended for a 12-month study were stored for 18 months. To simulate storage conditions for DBS used on a daily basis as QC materials [4,6], some DBS stored at 4 °C were removed every weekday and allowed to warm to room temperature; the mylar zip-closure bag was opened for several minutes and a DBS specimen was removed and stored at −80 °C. This working bag study was conducted for 45 days. The high-humidity study (also with DBS specimen removal every weekday) was conducted for 53 days.

HPLC–ESI-MS/MS analysis

At the completion of stability studies the DBS specimens were removed from −80 °C storage, allowed to warm to room temperature, and each specimen was analyzed in triplicate. The throughput of a negative ion-mode HPLC–ESI-MS/MS method to detect C26:0-LPC in DBS [7] was improved to achieve an HPLC analysis time of 1 min per sample by increasing the mobile phase flow rate to 0.45 mL/min. The isocratic mobile phase was 50:50 methanol/acetonitrile containing 5 mM ammonium acetate. Ion source parameters were optimized for this higher flow rate by increasing Gas 1 (desolvation gas) to 35, Gas 2 (heater gas) to 20, and the temperature to 400 ° C. Analyte and internal standard were monitored with the MRM pairs 620.6 → 395.3 and 624.6 → 399.3, respectively. The C26:0-LPC was quantitated by the following calculation [8–10]: (C26:0-LPC peak area/$^{2}H_{4}$-C26:0-LPC peak area) × [$^{2}H_{4}$-C26:0-LPC] × 14.29. The [$^{2}H_{4}$-C26:0-LPC] in extraction solution was 0.2 μM, and 14.29 = 100/7, which represents the dilution of blood from a 3/16″ punch [11] in 100 μL of extraction solution. For the stability studies at different temperatures results are shown (Table 1) as the fraction of remaining C26:0-LPC relative to C26:0-LPC in samples stored at −80 °C for the duration of the stability study. This fraction is the quotient of the y-intercept (from linear regression) and the concentration of C26:0-LPC measured in samples stored at −80 °C for the duration of the stability study.

Results

Quality control materials

Zero enrichment QC material was characterized as 0.03 μM C26:0-LPC (95% confidence interval 0.01 to 0.05 μM), 1 μM enriched QC material was characterized as 0.98 μM (95% confidence interval 0.85 to 1.11 μM), and 10 μM enriched QC material was characterized as 9.21 μM (95% confidence interval 8.15 to 10.27 μM).

Stability studies

Linear regression of the C26:0-LPC quantities in DBS stored with desiccant for at least 6 months resulted in small slopes at 4 temperatures [−20 °C (0.0003), 4 °C (−0.0005), ambient temperature (−0.001), and 37 °C (−0.0029)]. Linear regression of the measured C26:0-LPC resulted in a slope of −0.0005 for DBS stored at 4 °C for 45 days with desiccant but removed daily from refrigeration with opening of their storage bag simulating daily use of QC materials. Storage at ambient temperature with relative humidity > 90% for 53 days resulted in a linear regression of C26:0-LPC with a slope of −0.0115. These results are summarized in Table 1.

Conclusions

The enriched C26:0-LPC was stable in DBS under all of the conditions tested, as evidenced by small slope values and fractions close to 1.00 for C26:0-LPC in samples stored at different temperatures compared to samples stored at −80 °C for the duration of the stability study. X-ALD QC materials are currently available on a pilot basis from the Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention.

Acknowledgments

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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Table 1

Stability study results for C26:0-lysophosphatidylcholine.

Storage conditions			Linear regression results	
Temperature, °C	**Desiccant**	**Time, days**	**Slope**	**Y-intercept/Day 0 measurement**
−20	Yes	171 and 518	0.0003 and 0.0002	0.93 and 1.05
4	Yes	171 and 518	−0.0005 and <0.0001	0.96 and 1.08
Ambient	Yes	171 and 518	−0.0010 and <0.0001	0.97 and 1.08
37	Yes	171 and 518	−0.0029 and <0.0001	1.10 and 0.96
4[a]	Yes	45	−0.0005	0.93
Ambient[b]	No	53	−0.0115	0.97

[a]Storage bag was warmed to ambient temperature and opened every weekday to simulate quality control material usage.

[b]Storage bag was not closed and maintained at >90% relative humidity.