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

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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-ALD¹) 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

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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 $^2\text{H}_4$ -hexacosanoyl lysophosphatidylcholine ($^2\text{H}_4$ -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 $50 \pm 1\%$ [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 $620.6 \rightarrow 395.3$ and $624.6 \rightarrow 399.3$, respectively. The C26:0-LPC was quantitated by the following calculation [8–10]: $(\text{C26:0-LPC peak area}/^2\text{H}_4\text{-C26:0-LPC peak area}) \times [^2\text{H}_4\text{-C26:0-LPC}] \times 14.29$. The $[^2\text{H}_4\text{-C26:0-LPC}]$ in extraction solution was $0.2\text{ }\mu\text{M}$, and $14.29 = 100/7$, which represents the dilution of blood from a $3/16''$ punch [11] in $100\text{ }\mu\text{L}$ 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 $0.03\text{ }\mu\text{M}$ C26:0-LPC (95% confidence interval 0.01 to $0.05\text{ }\mu\text{M}$), $1\text{ }\mu\text{M}$ enriched QC material was characterized as $0.98\text{ }\mu\text{M}$ (95% confidence interval 0.85 to $1.11\text{ }\mu\text{M}$), and $10\text{ }\mu\text{M}$ enriched QC material was characterized as $9.21\text{ }\mu\text{M}$ (95% confidence interval 8.15 to $10.27\text{ }\mu\text{M}$).

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

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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	

^aStorage bag was warmed to ambient temperature and opened every weekday to simulate quality control material usage.

^bStorage bag was not closed and maintained at >90% relative humidity.