Clinical Biochemistry 49 (2016) 161–165

Contents lists available at ScienceDirect

Clinical Biochemistry

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

Simultaneous quantitation of hexacosanoyl lysophosphatidylcholine, amino acids, acylcarnitines, and succinylacetone during FIA–ESI–MS/MS analysis of dried blood spot extracts for newborn screening

Christopher A. Haynes *, Víctor R. De Jesús

Biochemical Mass Spectrometry Laboratory, Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention, MS F19, Atlanta, GA 30341, USA

ARTICLE INFO

Article history:
Received 19 June 2015
Received in revised form 25 September 2015
Accepted 28 September 2015
Available online 1 October 2015

Keywords:
Flow-injection analysis
Tandem mass spectrometry
Newborn screening
Dried blood spot
X-linked adrenoleukodystrophy

ABSTRACT

Objectives: The goal of this study was to include the quantitation of hexacosanoyl lysophosphatidylcholine, a biomarker for X-linked adrenoleukodystrophy and other peroxisomal disorders, in the routine extraction and analysis procedure used to quantitate amino acids, acylcarnitines, and succinylacetone during newborn screening. Criteria for the method included use of a single punch from a dried blood spot, one simple extraction of the punch, no high-performance liquid chromatography, and utilizing tandem mass spectrometry to quantitate the analytes.

Design and methods: Dried blood spot punches were extracted with a methanolic solution of stable-isotope labeled internal standards, formic acid, and hydrazine, followed by flow injection analysis–electrospray ionization–tandem mass spectrometry.

Results: Quantitation of amino acids, acylcarnitines, and hexacosanoyl lysophosphatidylcholine using this combined method was similar to results obtained using two separate methods.

Conclusions: A single dried blood spot punch extracted by a rapid (45 min), simple procedure can be analyzed with high throughput (2 min per sample) to quantitate amino acids, acylcarnitines, succinylacetone, and hexacosanoyl lysophosphatidylcholine.

1. Introduction

Newborn screening (NBS)[1] utilizes the analysis of dried blood spot (DBS) extracts for biomarkers [e.g. amino acids (AA) and acylcarnitines (AC)] that indicate an inborn error of metabolism when they are outside normal limits (elevated or deficient) [1]. Early detection, diagnosis, and treatment of inborn errors of metabolism prevent disability and death [2]. Flow injection analysis–electrospray ionization–tandem mass spectrometry (FIA–ESI–MS/MS) is the most widely used method to quantitate AA and AC in DBS extracts because it is rapid, sensitive, specific, and easily multiplexed for 20 to 30 analytes of interest and their corresponding stable-isotope labeled internal standards used for quantitation [3–5].

A variety of DBS extraction procedures have been published and/or incorporated into commercial products [5–7]. Criteria for a widely applicable procedure include simplicity, rapidity, avoiding expensive or hazardous reagents, and the ability to extract biomarkers with a variety of chemical properties [e.g. AA, AC, succinylacetone (SUAC), and lipids] from the DBS punch. Some methods derivatize AA, AC, and SUAC to form butyl esters [8] while other methods do not [9]. A hydrazine derivatization of SUAC is commonly used to improve its positive-mode ionization efficiency [5].

We describe a DBS extraction (without derivatization to form butyl esters) and FIA–ESI–MS/MS analytical method which incorporates quantitation of hexacosanyl lysophosphatidylcholine (C26:0-LPC) into routine analysis of AA, AC, and SUAC. C26:0-LPC is a biomarker for X-linked adrenoleukodystrophy (X-ALD) [10] and other peroxisomal biogenesis disorders (PBD). It has previously been analyzed by FIA–ESI–MS/MS [11] and high-performance liquid chromatography–ESI–MS/MS (HPLC–ESI–MS/MS), both of which typically require a second punch from the newborn's DBS sample, a separate extraction protocol, and a separate analysis with respect to AA–AC–SUAC quantitation [12–14]. We used the method described here to quantitate AA, AC, SUAC, and C26:0-LPC in quality control DBS specimens produced at the Centers for Disease Control and Prevention's (CDC) Newborn Screening Quality Assurance Program (NSQAP). These quality control DBSs were prepared from human blood enriched with AA, AC, and SUAC followed

* Corresponding author.
E-mail address: cph7@cdc.gov (C.A. Haynes).

[1] Abbreviations: NBS (newborn screening), DBS (dried blood spot), AA (amino acids), AC (acylcarnitines), FIA–ESI–MS/MS (flow injection analysis–electrospray ionization–tandem mass spectrometry), SUAC (succinylacetone), C26:0-LPC (hexacosanoyl lysophosphatidylcholine), X-ALD (X-linked adrenoleukodystrophy), PBD (peroxisomal biogenesis disorder), HPLC–ESI–MS/MS (high performance liquid chromatography–electrospray ionization–tandem mass spectrometry), NSQAP (Newborn Screening Quality Assurance Program), SED (single-enzyme defect), DBP (D-bifunctional protein deficiency), MRM (multiple reaction monitoring), R4S (Region IV Stork MS/MS Collaborative Project).

http://dx.doi.org/10.1016/j.clinbiochem.2015.09.011
0009-9120/

by homogeneity testing and characterization of AA, AC, and SUAC quantities via 20 independent measurements [15]. The analyte enrichment of human blood utilized commercially available standards prepared as stock solutions (in water or methanol) and their concentrations were verified by comparison of diluted analyte peak areas to commercially available stable-isotope labeled AA, AC, and SUAC. Our results indicated that the recoveries of AA, AC, and C26:0-LPC were similar to the results obtained from two separate methods (AA–AC–SUAC [5] and C26:0-LPC [14]), and that the quantitation of SUAC was slightly less sensitive than NSQAP's in-house AA–AC–SUAC method. Anonymous residual DBS specimens from presumed normal newborns (N = 151) were assayed to determine the mean and 95% confidence interval of C26:0-LPC, and to confirm that AA, AC, and SUAC quantitation was similar to aggregate NBS data from the Region 4 Stork MS/MS Collaborative (R4S) [4,16]. In addition, analysis of DBS specimens from confirmed cases (N = 28) of single-enzyme defect (SED), D-bifunctional protein deficiency (DBP) and PBD/Zellweger spectrum disorder showed C26:0-LPC concentrations higher than the 95% confidence interval for presumed normal newborns in every case.

2. Materials and methods

2.1. Reagents

D_4-C26:0-LPC was from Avanti Polar Lipids (Alabaster, AL), and stable-isotope labeled AA, AC, and SUAC were from Cambridge Isotopes (Tewksbury, MA). HPLC-MS grade water, methanol, and formic acid were from Fisher Scientific (Pittsburg, PA). Hydrazine hydrate was from Sigma-Aldrich (St. Louis, MO).

2.2. Dried blood spots

Two specimens were quality control materials enriched with AA, AC, and SUAC (1432 and 1434), three specimens were quality control materials enriched with C26:0-LPC (13104, 13105, and 13106), seven specimens were calibrators enriched with SUAC (Cal A to Cal G), 151 specimens were anonymous residual newborn screening punches (NBS 1 to NBS 151), and 28 specimens were from confirmed cases of SED, DBP, and PBD. All punches were 3 mm (1/8″). The blood used to prepare the quality control and calibrator specimens was hematocrit adjusted to 50 ± 1% and either lysed by freeze-thawing (1432, 1434, 13104, 13105, and 13106) or used with intact red-blood cells (SUAC calibrators). Lysed blood DBS were 100 μL each and intact blood DBS were 75 μL each; all NSQAP DBS were prepared on Whatman 903 paper, dried overnight, and stored at −20 °C with low (<30%) humidity. The anonymous, residual NBS DBS were stored at −20 °C with low (<30%) humidity for 29 months before analysis, and the confirmed peroxisomal disorder DBS were stored at −20 °C with low (<30%) humidity for 7 years before analysis.

2.3. Internal standards

A stock solution of $^{13}C_5$-succinylacetone (1 mg/mL) was prepared in water, aliquoted, and stored at −70 °C; 79.6 μL of this stock was added to 200 mL of methanol for a final concentration of 2.5 μM. A stock solution of D_4-C26:0-LPC (1 mg/mL) was prepared in methanol and stored at 4 °C; 184.4 μL of this stock was added to the same 200 mL of methanol for a final concentration of 0.2 μM. One vial each of NSK-A, NSK-B, and NSK-BG was solubilized according to the manufacturer's instructions and added to the 200 mL of methanol for final concentrations of 2.5 μM [phenylalanine (Phe), leucine (Leu), methionine (Met), tyrosine (Tyr), valine (Val), citrulline (Cit), arginine (Arg), and alanine (Ala)], 0.76 μM [free carnitine (C0)], 0.04 μM [acetylcarnitine (C2), propionylcarnitine (C3), butyrylcarnitine (C4), isovalerylcarnitine (C5), hydroxyisovalerylcarnitine (C5OH), octanoylcarnitine (C8), glutarylcarnitine (C5DC), dodecanoylcarnitine (C12), and tetradecanoylcarnitine (C14)], and 0.08 μM [palmitoylcarnitine (C16) and octadecanoylcarnitine (C18)]. The internal standard solution also contained 100 μL (0.05%) formic acid and 30 μL hydrazine hydrate (2.7 mM hydrazine) in order to derivatize SUAC to a hydrazone and improve its positive ion mode peak intensity. The internal standard solution was stored at 4 °C, allowed to warm to room temperature before use, and discarded after 10 days.

2.4. Sample extraction

Each DBS punch (3 mm) was placed in a well of a polypropylene 96-well plate (VWR, Suwannee, GA), 100 μL of the methanolic internal standard solution was added to each well, the plate was heat-sealed with foil (BioSero, San Diego, CA), and extracted for 45 min at 45 °C with shaking in a covered incubator (Thermo Scientific, Waltham, MA). After extraction the liquid contents of each well were transferred to a new 96-well plate followed by heat-sealing and FIA–ESI–MS/MS analysis.

2.5. Flow-injection analysis by electrospray ionization tandem mass spectrometry

An Agilent 1290 liquid chromatography system was used with an Applied Biosystems API-4000 triple quadrupole mass spectrometer to analyze the DBS punch extracts in positive ion mode. Turbo V ion source conditions were curtain gas = 20, gas 1 = 30, gas 2 = 10, electrospray capillary voltage = 5.5 kV, and temperature = ambient. The collision gas setting was 5. Each sample injection was 10 μL. The mobile phase was methanol containing 0.02% formic acid, and the mobile phase flow rate was reduced during each analysis to increase the residence time of analytes in the ion source, thereby improving the number of scans with high signal-to-noise obtained per injection. Specifically, the flow rate was 100 μL per min at sample injection, slowed to 18 μL per minute between 0.1 and 1.1 min, increased to 400 μL per min between 1.1 and 1.5 min, and returned to 100 μL per min between 1.5 and 2.0 min. The mass spectrometer acquired data between 0 and 2 min by performing 5 experiments: 1) multiple reaction monitoring (MRM) for C0, C2, C3, and their internal standards, 2) precursor ion scanning from *m/z* 215 to 475 for precursors of *m/z* 85.0 (C3 to C18OH AC's), 3) neutral loss scanning from *m/z* 74 to 220 for neutral loss of 46.0 Da (non-basic AA's), 4) MRM for Arg, Cit, SUAC, and their internal standards, and 5) MRM for C26:0-LPC and its internal standard. The MRM transitions used in the 5th experiment were 636.5 → 104.1 (C26:0-LPC) and 640.5 → 104.1 (D_4-C26:0-LPC). Supplementary Table 1 shows scan parameters for all analytes.

2.6. Data processing

The data files from Analyst (1.5.1) were processed by ChemoView (Applied Biosystems, Foster City CA). The concentration of each analyte was calculated using the formula:

(analyte peak area, cps) / (internal standard peak area, cps) × [internal standard, μM] × dilution factor.

Dilution factor was 32.26, which represents the volume of the extraction solution (100 μL) divided by the volume of whole blood at 50% hematocrit in a 3 mm punch (3.1 μL [17]).

3. Results

3.1. Quality control materials for amino acids, acylcarnitines, and succinylacetone

The quantitative results of analysis by the method described here were compared to the results of NSQAP's in-house AA–AC–SUAC analysis [5]. Table 1 shows the mean and 95% confidence interval for these analytes as measured by the two methods using NSQAP's quality control