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Proficiency testing outcomes of 3-hydroxyisovalerylcarnitine measurements by tandem mass spectrometry in newborn screening

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ABSTRACT

Background: The use of tandem mass spectrometry (MS/MS) for the analysis of amino acids and acylcarnitines from dried-blood spots (DBS) has become routine practice in newborn screening laboratories. The Newborn Screening Quality Assurance Program (NSQAP) added 3-hydroxyisovalerylcarnitine (C5OH) into its routine quality control and proficiency testing (PT) DBS materials for MS/MS to assure the quality of C5OH screening. We report the results from NSQAP evaluations for C5OH-enriched DBS, and summarize participant screening practices based on their analytical methods.

Methods: NSQAP prepared C5OH-enriched DBS materials for its participants. Laboratories reported quantitative and qualitative results. Bias plots of quantitative results were constructed using reported data and the results were sorted by an analytical method.

Results: NSQAP participants reported PT specimen 3964 as outside of normal limits for C5OH. The mean C5OH value for derivatized and non-derivatized methods was 2.80 and 2.67 μmol/l, respectively. Reported data from other specimens showed a similar trend in derivatized vs. non-derivatized assay results. Differences in C5OH quantitative values were observed among laboratories using different internal standards.

Conclusions: C5OH MS/MS measurements in DBS assays varied by method and the choice of internal standards. The use of NSQAP's DBS materials allows harmonization of C5OH measurements by newborn screening laboratories worldwide.

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1. Introduction

Analysis of amino acids and acylcarnitines by tandem mass spectrometry (MS/MS) has become the method of choice for screening newborns for amino acid and organic acidemias, and fatty acid oxidation disorders [1–4]. The accumulation of acyl-CoA which esterifies to acylcarnitines by carnitine acyltransferase serves as an excellent route to monitor increases of acylcarnitines in organic acidemias and fatty acid oxidation disorders. The accumulation of 3-hydroxyisovaleryl-CoA or 3-hydroxy-2-methylbutyryl-CoA may be indicative of low enzymatic activities and/or maternal conditions, and results in the elevation of plasma and urine concentrations of 3-hydroxyisovalerylcarnitine (C5OH) or 3-hydroxy-2-methylbutyryl-carnitine (C5OH-M, an isomer of C5OH) [5].

3-Hydroxyisovalerylcarnitine (C5OH) is a biomarker for screening of several inborn errors of metabolism, including multiple carboxylase deficiency (MCD), 3-hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA) deficiency, 3-methylcrotonyl-CoA carboxylase (3MCC) deficiency, and 3-methylglutaconyl-CoA hydratase deficiency. C5OH-M, an isomer of C5OH, is a biomarker for β-ketothiolase deficiency [5–9]. These disorders are identified in the American College of Medical Genetics' (ACMG) recommended uniform panel of conditions for inclusion by all state newborn screening programs in the U.S. [10]. To date all states have expanded to include at least the 29 core biochemical tests. As a result, quality assurance (QA) materials that include C5OH in dried-blood spots (DBS) are needed to monitor accurate identification and quantitation of this important biomarker.

The Newborn Screening Quality Assurance Program (NSQAP) at the Centers for Disease Control and Prevention (CDC) added C5OH to its DBS proficiency testing (PT) panels as well as its quality control materials. Here we report the 2009 results from NSQAP evaluations for 3 C5OH enriched DBS from our first PT challenges. In addition, we summarize participants' responses to C5OH screening practices based on their choice of internal standards and analytical methods.

2. Materials and methods

2.1. DBS specimen preparation

Whole blood collected in citrate phosphate dextrose adenine (CPDA-1) anticoagulant was purchased from Tennessee Blood Services

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(Memphis, TN, USA) and used to prepare the DBS materials. All DBS materials for PT were made from whole blood and adjusted to 50% hematocrit by plasma removal by previously described methods [11,12]. Blood pools were applied to Whatman® Grade 903 (Lot # W071) filter paper (Whatman, Inc. Piscataway, NJ), in 75 µl aliquots and dried overnight at ambient temperature. DBS materials were stored in zip-closure, low-gas permeable plastic bags (Com-Pac International, Carbondale, IL) with desiccant packets to ensure that humidity remained <30%. The stored DBS materials were transferred to zip-closure Mylar foil bags before addition of fresh desiccant packets prior to distribution to participating laboratories for analysis [13,14].

Four sets of DBS materials enriched with C5OH were sent in 2009 for independent evaluation by selected laboratories. The first DBS set consisted of five blind-coded, dose–response PT DBS materials were sent to five laboratories. These pilot PT samples were enriched with C5OH at 0, 1.5, 2.0, and 2.5 µmol/l to create a dose–response series. Three additional sets of DBS PT materials, with each set containing one C5OH-enriched specimen, were sent to the following: 1) 72 participating North American laboratories (April 2009) specimen 9225 with 4.12 µmol/l of C5OH; 2) 198 laboratories participating in NSQAP's acylcarnitine PT program (July 2009) specimen 3964 with 3.34 µmol/l of C5OH, and; 3) 204 laboratories participating in NSQAP's acylcarnitine PT program (October 2009) specimen 4965 with 2.09 µmol/l of C5OH.

2.2. Reagents

Unlabeled and stable isotope-labeled C5OH ($^2H_3$-hydroxyisovaleryl carnitine), and stable isotope-labeled acylcarnitine internal standards kit NSK-B were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). HPLC-grade acetonitrile, methanol, formic acid and water were from Fisher Scientific (Fairlawn, NJ) and used as received. Butanolic-HCl (3N) was purchased from Regis Technologies (Morton Grove, IL).

2.3. Specimen preparation for MS/MS

All samples were prepared in duplicate using a single 1/8″ (3.2 mm) diameter filter paper disk punch individually placed into 96-well polypropylene microtiter plates. DBS punches were extracted with 100 µl of methanolic solution containing stable isotope-labeled acylcarnitines and C5OH internal standards for 30 min at an ambient temperature with gentle agitation. The methanolic eluates (90 µl) were transferred to new 96-well plates for derivatization into butyl esters (BE), and dried under a gentle flow of heated (50 °C) nitrogen for 15 min. Then, 50 µl of 3 mol/l butanolic HCl was added to each well, tightly covered, and incubated for 15 min at 65 °C. After incubation, the plates were dried using heated (50 °C) nitrogen for 15 min, and then reconstituted prior to MS/MS analysis with 100 µl of acetonitrile, water, and formic acid solution (50:50:0.02% by volume).

2.4. Mass spectrometric analysis

In-house electrospray ionization (ESI) analysis on MS/MS was performed using a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA), equipped with an Acquity UPLC system (Waters, Milford, MA). Nitrogen was used as the nebulizing gas and argon was used as the collision gas at a pressure of 0.15 Pa. The source temperature was 120 °C and the capillary voltage used was 3.5 kV. The C5OH was analyzed by a precursor ion scan of m/z 85 (Pre 85) in positive ion mode. The cone voltage was held at 30 V and collision energy was 22 eV. The BE parent ions of interest were m/z 318 for C5OH, m/z 321 for $^2H_3$-hydroxyisovaleryl carnitine (d3-C5OH), m/z 311 for $^2H_9$-isovaleryl carnitine (d9-C5) and m/z 347 for $^2H_3$-octanoyl carnitine (d3-C8). Quantification of acylcarnitines was achieved by calculation of the ion abundance ratios of the unlabeled compound relative to each chosen stable isotope-labeled internal standard [15,16].

2.5. Statistical methods

The Fisher's Least Significant Difference (LSD) multiple comparison approach was chosen, using the Kruskal–Wallis test as the omnibus test before proceeding with pairwise comparisons. Means and standard deviations are reported for each method separately, as well as for an aggregate of the methods. A Wilcoxon Rank Sum test was used to compare the mean between any two methods, and a Kruskal–Wallis test was used to compare the mean between more than 2 methods. A p-value of ≤ 0.05 was considered statistically significant. A generalized extreme studentized deviate outlier detection algorithm was applied to the results from the 166 laboratories that reported specimen 3964 as outside of normal limits for C5OH and 168 laboratories that reported specimen 4965 as outside of normal limits for C5OH [17]. The algorithm was run separately for each aggregated analytical method and for each C5OH-enriched specimen. Outliers were removed from the analysis. An analysis was performed using all the data (i.e. no outliers removed), and the conclusions with respect to the significant differences reported in this study remained unchanged (results not shown).

3. Results

3.1. Pilot PT survey using a dose–response series

Five laboratories reported data for the pilot dose–response C5OH PT specimens. Two laboratories reported quantitative values using d3-C8 as the internal standard, and three laboratories reported quantitative values using d9-C5 as the internal standard. Each laboratory recovered a consistent fraction of enriched C5OH across the concentration range tested (0 to 2.5 µmol/l). The overall means of reported C5OH concentrations were comparable to each other, but reported C5OH values from one participant who used a non-derivatized (free acid, FA) method were below the others who used the butyl ester (BE) derivatization method (Fig. 1). BE method users with d9-C5 as the internal standard showed higher recovery (92%) compared to BE method users who used d3-C8 as the internal standard (85%). The FA method user recovered 53% of the expected value.

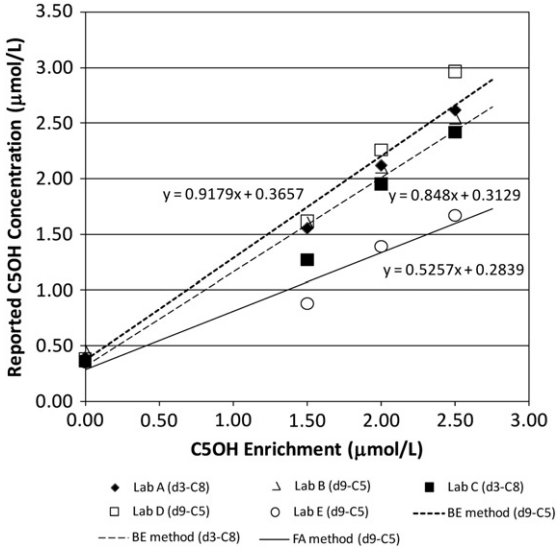

Fig. 1. Results from C5OH PT Pilot Survey, NSQAP Quarter 1 2009. Labs A–D reported using a butyl ester (BE) derivatization method and Lab E reported using a non-derivatized (FA) method.