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A novel method for quantitation of acylglycines in human dried blood spots by UPLC-tandem mass spectrometry

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ABSTRACT

Background: Several acylcarnitines used as primary markers on dried blood filter papers (DBS) for newborn screening lack specificity and contribute to a higher false positive rate. The analysis of urine acylglycines is useful in the diagnosis of inborn errors of metabolism (IEM) including medium chain acyl-CoA dehydrogenase deficiency (MCADD), isovaleric acidemia, and beta-ketothiolase deficiency (BKTD). Currently, no method for analyzing acylglycines from DBS has been published.

Methods: Acylglycines were extracted from two 3.2 mm DBS punches and butylated using Butanol-HCl. Ultra Performance Liquid Chromatography (UPLC-MS/MS) with run time of 10 min permits resolution and quantitation of 15 acylglycines; including several isobaric. Method development was completed. Reference intervals (n = 573) were established for four birth weight groups. Furthermore, samples from patients with a confirmed IEM (n = 11), and false positive screens (n = 78) were analyzed to validate the interpretation obtained from the newly established reference intervals.

Results: Calibration curves were linear from 0.005 to 25.0 μM. Ion suppression was evaluated as minimal (2 to 10%). Samples from known patients were used to validate the reference intervals. For C5OH-related disorders, tiglylglycine (TG), TG/acetylglycine (AG) ratio, 3methylcrotonylglycine (3MCG) and 3MCG/AG ratio increased specificity. Propionylglycine (PG) and PG/acetylglycine ratio were two discriminatory markers in the investigation of C3-related disorders. Hexanoylglycine (HG), octanoylglycine (OG), suberylglycine (SG), and the ratios HG/AG, OC/AG and SG/AG were excellent markers of MCADD deficiency.

Conclusion: This method shows potential application as a second tier screen in order to reduce the false positive rate for a number of IEM targeted by newborn screening.

1. Introduction

Inborn errors of metabolism (IEM) involving both fatty acids and amino acids lead to increased conjugation of acyl-coenzyme (acyl-CoA) intermediates with carnitine and glycine, resulting in their accumulation in both urine and blood [1–5]. Since the advent of newborn screening by tandem mass spectrometry in the early 1990s on dried blood spots (DBS) [4,6], the ability to measure numerous compounds (such as amino acids, acylcarnitines and acylglycines) has progressed with improved technology [7–9] such as liquid chromatography tandem mass spectrometry (LC-MS/MS).

The analysis of acylcarnitines in blood by tandem mass

Abbreviations: 2MBG, 2-methylbutyrylglycine; 3MCC, 3-methylcrotonyl-CoA carboxylase deficiency; 3MCG, 3-methylcrotonylglycine; AG, acetylglycine; BG, butyrylglycine; BKTD, beta ketothiolase deficiency; C3, propionylcarnitine; C5OH, 3-hydroxyisovalerylcarnitine; Cblc, cobalamin C deficiency; CG, cinnamoylglycine; DBS, dried blood spots; HG, hexanoylglycine; IEM, inborn errors of metabolism; IBG, isobutyrylglycine; IVA, isovaleric acidemia; IVG, isovalerylglycine; LC-MSMS, liquid chromatography tandem mass spectrometry; LLOQ, lower limit of quantitation; LOD, limit of detection; MCADD, medium chain acyl-CoA dehydrogenase deficiency; NA, not affected; Navail, not available; OG, Octanoylglycine; PA/MMA, propionic/methylmalonic acidemias; PG, propionylglycine; PPG, phenylpropionylglycine; PVG, pivaloylglycine; SG, suberylglycine; TG, tiglylglycine; UPLC, Ultra Performance Liquid Chromatography; VG, valerylglycine

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spectrometry is the current standard for screening and diagnosis of certain IEMs. Such diseases include medium chain acyl-CoA dehydrogenase deficiency (MCADD), isovaleric acidemia (IVA), 3-methylcrotonyl-CoA carboxylase deficiency (3MCC) and the propionic/methylmalonic acidemias (PA/MMA) [4]. Acylglycines are formed in mitochondria with acyl-CoA esters conjugating with glycine through the catalyzing enzyme glycine *N*-acyltransferase, and are useful urine markers for the diagnosis of these diseases [1–3].

Several primary screening markers such as propionylcarnitine (C3-marker for PA/MMA) and 3-hydroxyisovalerylcarnitine (C5OH-marker for 3MCC) lack specificity in identifying a number of clinical entities [6]. As a result, second tier screening for more specific markers, done on the original DBS sample of potentially screen positive babies, has improved screening performance for many disorders [10]. Any baby who is screen positive requires confirmatory diagnostic testing on new physiological samples (plasma, EDTA blood, urine, etc.). Diagnostic testing for the above disorders includes urine organic acids, plasma acylcarnitines, urine acylglycines and, when the former tests are abnormal, molecular or complementation testing.

Currently, there is no published methodology to quantify acylglycines in plasma or DBS. Here, we describe a novel method applying Ultra Performance Liquid Chromatography (UPLC-MS/MS) for the quantification of butylated acylglycines in DBS. We evaluated the reference intervals for acylglycine markers, including ratios, based on birth weight of screen negative control samples. We also analyzed DBS of screen positive babies that were subsequently found to be affected (true positive) or not affected (false positive), to validate the reference intervals. This method could be incorporated into newborn screening to improve screening performance and reduce false positive rates.

2. Materials and methods

2.1. Materials

H.J. ten Brink provided all acylglycines (www.vumc.com/branch/clinical-chemistry1/). This includes non-labelled and labelled acylglycines. The non-labelled compounds were acetylglycine (AG), propionylglycine (PG), isobutyrylglycine (IBG), butyrylglycine (BG), 3-methylcrotonylglycine (3-MCG), tiglylglycine (TG), 2-methylbutyrylglycine (2-MBG), isovalerylglycine (IVG), valerylglycine (VG), pivaloylglycine (PVG), hexanoylglycine (HG), octanoylglycine (OG), phenylpropionylglycine (PPG), cinnamoylglycine (CG), and suberylglycine (SG).

The stable isotope labelled compounds were [d_3]acetylglycine, [3,3,3-d_3]- propionylglycine, [4,4,4-d_3]butyrylglycine, [d_7]isobutyrylglycine, (D,L)-[d_9]2-methylbutyrylglycine, 3-methylcrotonyl-[$^{13}C_2$]glycine, [d_9]isovalerylglycine, [6,6,6-d_3]hexanoylglycine, [d_5]phenylpropionylglycine, and [2,2,7,7-d_4]suberylglycine.

Methanol and acetonitrile were high-performance liquid chromatography (HPLC) grade from Honeywell Burdick & Jackson® (Muskegon, Mi, USA). Formic Acid (≥98%) was from Sigma-Aldrich (St. Louis, Mo, USA), and 3.0 N HCl in *n*-butanol was from PerkinElmer Life and Analytical Sciences, Wallac Oy (Turku, Finland). Type-1 water was from Millipore Direct- Q3 UV water purification system (Darmstadt, Germany).

2.2. Samples

Blood spot samples from 573 babies (collected < 7 days of age) who were initially screen negative for all disorders were used as control samples to establish reference intervals for acylglycines and relevant analyte ratios. This group was further broken down into four groups (G), based on birth weight, to minimize the risk of false positive levels of acylglycines in preterm and low birth weight infants. The groups included: G1: < 1000 g N = 123, G2: 1000–1499 g N = 150, G3: 1500–2499 g N = 150, and G4: ≥2500 g N = 150.

Samples from 125 screen positive babies (collected < 7 days of age) were analyzed to validate the established reference intervals for acylglycines and ratios. As part of the diagnostic work-up, these babies were referred for diagnostic testing subsequent to their positive newborn screen. Diagnostic testing included urine organic acids, plasma acylcarnitines, urine acylglycines and, when the former tests are abnormal, molecular or complementation testing. The testing confirmed: MCADD (n = 3) [patient 1 homozygous for c.985 A > G in *ACADM*; patient 2 heterozygous c.734C > T and c.536_568 del33 in *ACADM*; patient 3 homozygous for c.984delG in *ACADM*], MCADD carriers (n = 4), MCADD-Non Affected [NA] (n = 20), PA (n = 1) [patient 1 homozygous c.990dupT in *PCCB*], cbl-C (n = 2) [patient 1 no molecular testing was performed, complementation studies confirmed *cblC* complementation; patient 2: no molecular or complementation was performed, older sibling was affected with CblC], MUT (n = 1) [patient 1: no molecular testing was performed, abnormal organic acids, acylcarnitines and older sibling affected with MMA-B12 responsive], maternal B12 deficiency (n = 7), elevated propionylcarnitine (C3)-NA (n = 21), BKTD (n = 2) [patient 1 homozygous c.1172 G > C (p.G391A) in *ACAT1*; patient 2 homozygous for c.369_372delCAAA in *ACAT1]*, 3MCC (n = 1) [patient 1 no molecular testing was performed, abnormal organic acids and acylcarnitines confirmed diagnosis], maternal 3MCC (n = 8), elevated 3-hydroxyisovalerylcarnitine (C5OH)-NA (n = 24), IVA (n = 1) [patient 1 homozygous c.1183C > T (p.R395X) in *IVD]*, and IVA-NA (n = 12).

Since the samples for acylglycine measurements were processed double-blinded, we also included for review screen negative > 2500 g (n = 47), and screen negative < 1000 g (n = 30) in the acylglycine runs.

2.3. Preparation of calibrators, quality controls and internal standards

Acylglycine stock solutions (non-labelled and labelled) were prepared in methanol. All stock solutions (10 mM) were stored at −20 °C. Tuning solutions from stock were prepared in water: acetonitrile (20:80) with a final working concentration of 5.0 μM.

DBS calibrators for all acylglycines combined were prepared by spiking whole blood (heparinised) from healthy adults with either methanol:water (50:50) working solutions or stock solutions at final concentrations of 0.005, 0.01, 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 2.50, 5.00, 10.00 and 25.00 μM.

The endogenous acylglycine levels present in the blood from healthy adults, prior to spiking were taken into account while designing the calibration curves. Most acylglycines were absent from the non-spiked blood. However for the following acylglycines (AG, 2MBG, CG, SG), the background (endogenous level) was added to the spiked amount. The final concentrations were used to design the calibration curve. The endogenous levels for AG, 2MBG, CG and SG were derived with the use of a factor. Briefly, regression analysis of calibration curves (using 12 calibrators) was used to obtain the slope and the y intercept. This factor represents the endogenous level, which was then added to the 12 calibrator levels, to achieve the final concentrations of 1.085, 1.09, 1.13, 1.18, 1.33, 1.58, 1.83, 2.08, 3.58, 6.08, 11.08 and 26.08 μM for AG; 0.115, 0.12, 0.16, 0.21, 0.36, 0.61, 0.86, 1.11, 2.61, 5.11, 10.11 and 25.11 μM for 2MBG; 0.085, 0.09, 0.13, 0.18, 0.33, 0.58, 0.83, 1.08, 2.58, 5.08, 10.08 and 25.08 μM for CG; and 0.185, 0.19, 0.23, 0.28, 0.43, 0.68, 0.93, 1.18, 2.68, 5.18, 10.18 and 25.18 μM for SG.

In-house DBS control samples prepared from heparinised whole blood pools from healthy adults and spiked with working solutions were set at two final concentrations for OG and PVG (0.3 and 1.5 μM) and at three concentrations for the remainder 13 acylglycines (0.8, 2.0 and 5.0 μM).

An intermediate internal standard mix (200 μM) stored at −20 °C and stored in 1.0 mL aliquots was prepared in methanol containing 200 μM each of [d_3]acetylglycine, [3,3,3-d_3]-propionylglycine, [4,4,4-d_3]butyrylglycine, [d_7]isobutyrylglycine, (D,L)-[d_9]2-methylbutyrylglycine, 3-methylcrotonyl-[$^{13}C_2$]glycine, [d_9]isovalerylglycine, [6,6,6-d_3]hexanoylglycine, [d_5]phenylpropionylglycine, and [2,2,7,7-