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Quantitation of gamma-hydroxybutyric acid in dried blood spots: Feasibility assessment for newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency

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ABSTRACT

Objective: SSADH deficiency, the most prevalent autosomal recessive disorder of GABA degradation, is characterized by elevated gamma-hydroxybutyric acid (GHB). Neurological outcomes may be improved with early intervention and anticipatory guidance. Morbidity has been compounded by complications, e.g. hypotonia, in undiagnosed infants with otherwise routine childhood illnesses. We report pilot methodology on the feasibility of newborn screening for SSADH deficiency.
Method: Dried blood spot (DBS) cards from patients affected with SSADH deficiency were compared with 2831 archival DBS cards for gamma-hydroxybutyric acid content. Following extraction with methanol, GHB in DBS was separated and analyzed using ultra high-performance liquid chromatography tandem mass spectrometry.
Results: Methodology was validated to meet satisfactory accuracy and reproducibility criteria, including intra-day and inter-day validation. Archival refrigerated dried blood spot samples of babies, infants and children ($N = 2831$) were screened for GHB, yielding a mean +/− S.D. of 8 ± 5 nM (99.9%-tile 63 nM) (Min 0.0 Max 78 nM). The measured mean and median concentrations in blood spots derived from seven SSADH deficient patients were 1182 nM and 699 nM respectively (Min 124, Max 4851 nM).
Conclusions: GHB concentration in all 2831 dried blood spot cards was well below the lowest concentration of affected children. These data provide proof-of-principle for screening methodology to detect SSADH deficiency with applicability to newborn screening and earlier diagnosis.

1. Introduction

Newborn screening for inherited metabolic disorders (IMDs) has undergone a paradigm shift since the seminal groundwork on phenylketonuria (PKU) by Robert Guthrie [1–3]. PKU remains the prototypical disorder amenable to mass newborn screening, based upon three key criteria set forth by early pioneers in the field: 1) prevalence (PKU ~ 1:10,000 live newborns); 2) treatment (dietary management); and 3) a high throughput, reliable method for detection (initially, bacterial inhibition assay for PHE; today, tandem mass spectrometry). Nonetheless, the technological advances in tandem mass spectrometry have resulted in screening for many IMDs that do not necessarily fulfill the above criteria, resulting in considerable debate about the prudence and justification of expanded newborn screening [4]. SSADH deficiency is a rare disorder, with approximately 450 individuals diagnosed [5]. Emerging clinical trials are in development based upon preclinical treatment efficacy data developed in the corresponding murine model, so-called *aldh5a1*$^{-/-}$ mice (aldh5a1 = aldehyde dehydrogenase 5a1 = succinic semialdehyde dehydrogenase). These trials include an ongoing open-label trial of taurine intervention (NCT01608178; www.clinicaltrials.gov) and a developing trial of the $GABA_B$ receptor antagonist SGS-742 [6]. These trials, and others in the early planning stages, have provided the rationale for exploratory studies focused on newborn screening for SSADH deficiency.

SSADH deficiency (or gamma-hydroxybutyric aciduria) is a rare autosomal recessively inherited defect in the catabolic pathway of GABA. In the absence of SSADH, transamination of GABA to succinic semialdehyde is followed by its conversion to 4-hydroxybutryic acid (gamma-hydroxybutyric acid, or GHB), the biochemical hallmark of the disease (Fig. 1). SSADH deficiency is typically a slowly progressive or static encephalopathy with late infantile to early childhood onset, presenting with ataxia, hypotonia, speech disturbance and variable

Abbreviations: SSADH, succinic semialdehyde dehydrogenase; DBS, dried blood spots; IMDs, inherited metabolic disorders; PKU, phenylketonuria; PHE, phenylalanine; GABA, gamma aminobutyric acid; GHB, gamma-hydroxybutyric acid; α-HBA, alpha-hydroxybutyric acid; β-HBA, beta hydroxybutyric acid; α-HisoBA, alpha isohydroxybutyric acid; β-HisoBA, beta isohydroxybutyric acid; UPLC, ultrahigh pressure liquid chromatography; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; MRM, multiple reaction monitoring.

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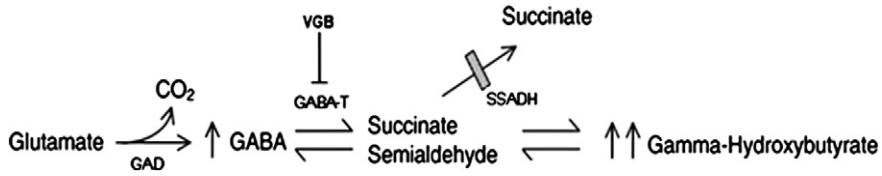

Fig. 1. Schematic pathway of GABA metabolism and the formation of GHB; Abbreviations: GAD, glutamic acid decarboxylase; GABA, γ-aminobutyrate; GABA-T, GABA transaminase; VGB, vigabatrin (suicide inhibitor of GABA-T and frequent intervention in SSADH-deficient patients); SSADH, succinic semialdehyde dehydrogenase. The block in patients is indicated by the cross-hatched box. Upward arrows indicate the relative increase in metabolite concentration in physiological fluids.

degrees of intellectual impairment [7,8]. MRI studies show a dentatopallidoluysian pattern. Seizures are present in half of patients and EEG shows background slowing and typically generalized spike-wave discharges [9]. Heterogeneity in this disorder is extensive, but neurological morbidity is a constant feature, and early targeted intervention holds the potential to mitigate this pathophysiology.

Despite more than a decade of evaluating disease pathology in *aldh5a1*$^{-/-}$ mice, we still lack a comprehensive picture of the underlying pathomechanisms in this disorder [10]. Nonetheless, the emerging data suggests that elevated GABA plays a major role in CNS neurotoxicity [6]. Studies in *aldh5a1*$^{-/-}$ mice have revealed metabolic dysfunction in developing mice [11]. Both GABA and GHB were consistently elevated in fetal extracts, as early as day 10 embryos, and both remained elevated until birth. These data underscore the potential for disturbed GABAergic circuitry in the developing *aldh5a1*$^{-/-}$ fetus, and support the notion that early intervention may mitigate long-term neurological deficits. With these concepts in mind, we have begun to explore the feasibility of newborn screening for SSADH deficiency in dried blood spots, outlined in the current report.

2. Materials and methods

2.1. Materials

GHB sodium salt was from Sigma-Aldrich, Saint Louis (MO) USA and 1 mg/mL d_6GHB sodium salt in methanol was purchased from Cerilliant, Round Rock, TX (USA).

Water, methanol and acetonitrile Optima Grade were purchased from Fisher Scientific, Pittsburgh (PA) USA. Formic acid ACS grade was from J.T. Baker, Phillipsburg, NJ (USA). Polypropylene 96 well plates were from Fisher Scientific.

2.2. Dried blood spot quality controls, calibrators and samples

Quality controls at low, medium and high concentration were prepared as follows: low quality control samples were pooled normal blood which had been hemolyzed and was spotted on dried blood spot cards. Medium control was whole blood spiked at the approximate concentration of 352 nM, spotted on dried blood spot cards. High quality control samples were dried blood spots from a patient with confirmed SSADH diagnosis. Quality control dried blood spot samples were stored at 4 °C with desiccant and analyzed at the beginning and end of each batch.

Calibrators for GHB were prepared by spiking 10 mL of pooled whole blood of healthy individuals with 100 μL of aqueous standard solutions prepared at the following concentrations: 0.9, 2.4, 4.8, 9.6, 24, 48, 72, 96 mM. Spiked blood at each concentration was spotted on dried blood spot cards (paper Whatman 903), dried overnight and stored at 4 °C with desiccant for later use. Calibrators were analyzed with each batch.

Blood samples from seven patients with SSADH deficiency, confirmed by persistent 4-hydroxybutyric aciduria and mutation analysis were also spotted on paper and analyzed to confirm the validity of the assay.

Archival DBS specimen cards (2831 archival specimens for supplemental newborn screening, a clinical fee for service test of babies, infants and children stored for about twelve months at 4 °C) were screened with the present method.

2.3. Sample extraction

Using a Wallac automated puncher three 1/8 in. disks for each blank, calibrator, control and sample were directly punched in each well of a 96 well polypropylene plate.

Two hundred microliters of methanol containing 500 ng/mL d_6 GHB internal standard was added in each well with the exception of two wells for solvent blank with and without paper. After 30 min at room temperature the extracts were transferred into another clean plate, dried under a gentle stream of nitrogen at 40 °C and reconstituted with 90 μL of water [11]. Fifteen microliters of aqueous extract was injected into the UPLC–MS/MS system in partial loop injection with needle overfill mode.

2.4. Instrumentation

A Xevo TQ MS™ UPLC–MS/MS system from Waters, Milford Massachusetts (USA) was used for this assay.

Ultrahigh pressure liquid chromatography was conducted on a T3 HSS Waters column 100 × 2.1 mm, 1.8 μm at 60 °C. The flow rate was 0.25 mL/min with a step gradient going from 3% to 98% acetonitrile with 0.1% formic acid using a divert valve for the first minute (3% acetonitrile for 2.8 min rapidly to 98% for 0.5 min followed by a 0.7 min equilibration). The total run time was 4 min including equilibration.

2.4.1. Mass spectrometry

Ions were formed by electro spray ionization in negative ion mode and detected with multiple reaction monitoring (MRM). Conditions were: capillary 2.00 kV, cone 28.00 V, extractor 3.00 V, source temperature 150 °C, desolvation temperature 450 °C, cone gas flow 20 L/h, desolvation gas flow 1000 L/h, collision gas flow 0.15 mL/min and collision energy 9 V.

MRM transitions were *m/z* 109 → 90 for d_6 GHB and *m/z* 103 → 85 for GHB. GHB also shares the following transitions *m/z* 103 → 57 and *m/z* 103 → 73 with its isomers α-hydroxybutyric acid (α-HBA), α-hydroxyisobutyric acid (α-HisoBA), β-hydroxybutyric acid (β-HBA), and β-hydroxyisobutyric acid (β-HisoBA). [12,13] Although these transitions are not used for quantitation they are acquired and monitored to ensure proper separation from GHB isomers (Figs. 2 and 3).

2.5. Quantification of GHB in dried blood spots

GHB in dried blood spots was quantified using the Targetlyx software from Waters. A calibration curve was determined using GHB/d_6GHB response at an increasing GHB concentration in dried blood spots. The response was linear from 8 to 1014 nM and r^2 was not less than 0.990 (weighed 1/x, extended through the origin).