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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 spots samples of babies, infants and children (N=2831) were screened for GHB, yielding a mean $\pm$ 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 4851nM).

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.

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Keywords

Succinic Semialdehyde Dehydrogenase Deficiency; Gamma-Hydroxybutyric Acid (GHB); Gamma-Aminobutyric Acid (GABA); Newborn Screening; UPLC-MS/MS; Rapid Neonatal Detection

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-hydroxybutyric acid (gamma-hydroxybutyric acid, or GHB), the biochemical hallmark of the disease (Figure 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 degrees of intellectual impairment [7,8]. MRI studies show a dentato-pallidolusian 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 bloodspots, 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 d₆ GHB sodium salt in methanol was purchased from Cerilliant, Round Rock, TX (USA). Water, methanol and acetonitrile Optima Grade were purchased from Fisher Scientific, Pittsburg (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 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 spots 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, 7, 96 mM. Spiked blood at each concentration was spotted on dried blood spots 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" 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 d₆ GHB internal standard was added in each well with the exception of two wells for solvent blank with and without paper. After thirty minutes 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 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 four minutes 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/Hr, desolvation gas flow 1000 L/Hr, 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 (Fig 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_6 GHB response at 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).

3 RESULTS

3.1 Validation of the method

Complete separation of GHB from isomers and other compounds was obtained using the T3 HSS UPLC column. This is a column compatible with highly aqueous mobile phases for the separation of very polar molecules. A few other columns were previously tested such as HILIC Silica and Amide, Waters Atlantis and Zorbax RRHD SB-C-18. Waters T3 HSS and Zorbax RRHD showed comparable and superior performance for this task: we chose to use T3HSS.

Ionization with ESI in positive and negative ion mode was tested: the negative mode was chosen due to a lower background noise. Ion suppression was qualitatively evaluated with the method of post column infusion. In particular the extract of a blank spot in duplicate from a dried blood spot card and the extract of two replicate dried blood spots were acquired while infusing a 19 nM aqueous solution of GHB (flow rate 5 μ L/min). Ion suppression was found to be negligible. Quantitative evaluation of ion suppression or absolute matrix effect was determined by comparison of the response area obtained from a fortified DBS extract at three different concentrations versus the response area of a standard at equal final concentration. Satisfactory accuracy and reproducibility criteria were achieved, including intra-day and inter-day validation (Table1).

The Whatman 903 paper contains an isobaric compound coeluting with GHB. Three different lots of paper available in our laboratory were tested and the paper used contained insignificant amounts. Therefore when a new type of card or format was processed, a blank taken from a clean corner of the card was analyzed to confirm that the interfering peak was of very minor contribution.

With this validated method GHB was measured in 2831 dried blood spot cards of babies, infants and children. GHB concentration was not age dependent as shown in supplemental figure 1.

Seven cards from patients with confirmed SSADH deficiency were analyzed. Mean GHB concentration in archival dried blood spots was 8 ± 5 nM (cut off at 99.9%-tile was 63 nM)

(Figure 4). The measured concentration in seven affected children were 124, 271, 609, 699, 715, 1000, 4851 nM, in each case well above the cut off Table 1.

4 Discussion/Conclusion

A reliable and effective procedure for determining GHB levels in dried blood spot cards was developed. Using this method, we were able to clearly distinguish between patients with a confirmed diagnosis of SSADH deficiency and the normal population of newborns and children. The cut off determined in archival dried blood spot cards was significantly less than the mean level in SSADH patients (1182 nM), as well as the lowest SSADH deficient patient (124 nM). Although the sample size of SSADH was small in this case (7 patients), due to the rareness of this condition, the method presented is valid and allows for the early diagnosis and potential treatment of SSADH deficiency in neonates. This would be clinically meaningful given that the mean age at diagnosis is two years and in some cases delayed until adulthood [14], by which time patients and families have typically already undergone diagnostic uncertainty if not a prolonged odyssey of testing.

Although the gold standard for diagnosis of SSADH deficiency centers on the molecular genetic analysis of the ALDH5A1 gene located on chromosome 6p [15], the current methodology would represent an important tool in the diagnostic armamentarium for this disorder, one in which samples could be readily obtained (heel stick, etc), readily (and inexpensively) transported and rapidly analyzed in specialist laboratories. Such testing could also replace diagnostic enzyme analysis in white cells derived from whole blood [16], a laborious method not routinely available in the majority of biochemical genetic diagnostic laboratories.

In some cases the developmental delay and hypotonia in SSADH deficiency have been associated with complications such as aspiration pneumonia from otherwise typical childhood respiratory illnesses. Newborn screening would allow for proper anticipatory guidance to reduce the incidence and severity of such problems, and will have a highlighted management role depending upon the outcome of clinical trials in progress. Although the current methodology is too laborious and slow to rapidly facilitate its interdigitation into current State newborn screening programs and platforms, it does provide a methodological baseline from which a more streamlined method may evolve in the near future. Ongoing efforts in the development of effective therapeutics for SSADH deficiency [6] should add support to the further development of newborn screening for SSADH deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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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Highlights

- Newborn screening for SSADH deficiency has been developed and validated
- Gamma-hydroxybutyrate (GHB) in dried blood spots (DBS) can be accurately quantified
- SSADH-deficiency can be differentiated from unaffected based upon GHB levels in DBS
- SSADH deficiency can be reliably documented in both DBS and urine organic acids

Figure 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.

Figure 2.

Chromatogram of GHB (1), α -HBA and α -HisoBA (2) and β -HBA and β -HisoBA (3) in pooled whole blood spiked with 48 nM GHB aqueous standard, dried on filter paper and extracted according to the validated method (refer to text for abbreviations).

Figure 3. Overlapping chromatograms of GHB in DBS of a healthy baby (GHB1), of a child affected by SSADH deficiency (GHB2) and internal standards (d₆GHB); intensity normalized to 2×10^5 .

Figure 4. GHB concentration distribution in 2831 archival newborn screening cards. GHB concentration is higher than 40 nM only in four subjects; the values are: 41, 49, 66 and 78 nM

Table 1

Method validation outcomes and concentration in children with SSADH deficiency

Linearity	from 8 to 1014 nM, $r^2 > 0.990$
Recovery	>70% area of GHB spiked before the extraction divided by area of GHB spiked after the extraction
Absolute matrix effect	<19% area of GHB spiked after the extraction divided by area of GHB standard
Intraday	Low Control 9 nM, 12% CV, Medium Control 90 nM, 5% CV, 90 % accuracy High Control 705 nM, 7% CV; 98% accuracy N= 6
Inter-day (22 days)	low control 19 nM CV 21.6%, medium control 352 nM CV 14.2%. high control 609 nM CV 15.1%.
2831 DBS screened	GHB mean and S.D. 8 ± 5 nM
Cut off	at 99.99 percentile 63 nM

SSADH Deficient Patients

N=7
 124, 271, 609, 699, 715, 1003, 4851 nM
 Mean 1182 (124-4851) **nM**