Targeted screening of succinic semialdehyde dehydrogenase deficiency (SSADHD) employing an enzymatic assay for γ-hydroxybutyric acid (GHB) in biofluids

C. Wernli, S. Finochiaro, C. Volken, H. Andresen-Streichert, A. Buettler, D. Gygax, G.S. Salomons, E.E. Jansen, G.R. Ainslie, K.R. Vogel, K.M. Gibson

**Article Info**

**Abstract**

Hypothesis: An enzymatic assay for quantification of γ-hydroxybutyric acid (GHB) in biofluids can be employed for targeted screening of succinic semialdehyde dehydrogenase deficiency (SSADHD) in selected populations. Rationale: We used a two-tiered study approach, in which the first study (proof of concept) examined 7 urine samples derived from patients with SSADHD and 5 controls, and the second study (feasibility study) examined a broader sample population of patients and controls, including plasma. Objective: Split samples of urine and plasma (anonymized) were evaluated by enzymatic assay, gas chromatography, and gas chromatography-mass spectrometry, and the results compared. Method: Multiple detection methods have been developed to detect GHB. We evaluated an enzymatic assay which employs recombinant GHB dehydrogenase coupled to NADH production, the latter quantified on a Cobas Integra 400 Plus. Results: In our proof of concept study, we analyzed 12 urine samples (5 controls, 7 SSADHD), and in the feasibility study we evaluated 33 urine samples (23 controls, 10 SSADHD) and 31 plasma samples (14 controls, 17 SSADHD). The enzymatic assay carried out on a routine clinical chemistry analyzer was robust, revealing excellent agreement with instrumental methods in urine (GC-FID: r = 0.997, p ≤ 0.001; GC–MS: r = 0.99, p ≤ 0.001); however, the assay slightly over-estimated GHB levels in plasma, especially those in which GHB levels were low. Conversely, correlations for the enzymatic assay with comparator methods for higher plasma GHB levels were excellent (GC–MS: r = 0.993, p ≤ 0.001). Conclusion: We have evaluated the capacity of this enzymatic assay to identify patients with SSADHD via quantitation of GHB. The data suggests that the enzymatic assay may be a suitable screening method to detect SSADHD in selected populations using urine. In addition, the assay can be used in basic research to elucidate the mechanism of the underlying disease or monitor GHB–levels for the evaluation of drug candidates. Synopsis: An enzymatic assay for GHB in biofluids was evaluated as a screening method for SSADHD and found to be reliable in urine, but in need of refinement for application to plasma.

© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

**Abbreviations:** CV, coefficient of variation; GABA, gamma-aminobutyric acid; GC-FID, gas chromatography-flame ionization detector; GC–MS, gas chromatography–mass spectrometry; GHB, gamma-hydroxybutyrate (also γ-hydroxybutyric acid); GHB-DH, GHB-dehydrogenase; IDDM, isotope dilution method; LOD, limit of detection; LLOQ, lower limit of quantitation; NADH, nicotinamide adenine dinucleotide, reduced form; r, correlation coefficient (Pearson); SSADHD, succinic semialdehyde dehydrogenase deficiency.

* Corresponding author at: University of Applied Sciences and Arts Northwestern Switzerland, School of Life Sciences, Institute for Chemistry and Bioanalytics, Gruendenstrasse 40, 4132 Muttenz, Switzerland.

E-mail address: cedric.wernli@fhnw.ch (C. Wernli).

1 Wernli C-Design of study, implementation of enzymatic assay, manuscript preparation, editing.
2 Finochiaro S, Volken C, Andresen-Streichert H, Buettler A, Gygax D-additional laboratory analyses, manuscript preparation, editing.
3 Salomons GS, Jansen EE-additional laboratory analyses, manuscript preparation, editing.
4 Ainslie CR, Vogel KR, and Gibson KM-secondary design of study, procurement of patient samples, manuscript preparation, editing.

http://dx.doi.org/10.1016/j.ymgmr.2016.07.009

2214-4269/© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

It is well established that GABA is the primary inhibitory neurotransmitter in CNS, where >1/3 of synapses employ it [1,2]. The GABA analogue, γ-hydroxybutyric acid (GHB), is present in mammalian CNS at ~1% the level of its parent compound. The exact role for GHB in CNS remains poorly defined [3]. GHB potentiates dopaminergic activity, is employed therapeutically for narcolepsy, and is abused as a recreational drug and agent to facilitate sexual assault [4–6]. The latter properties have spurred interest in numerous toxicological settings for methods enabling rapid detection of GHB in biofluids, yet such assays are challenging because of the short $t_{1/2}$ for GHB of about 30–50 min [7].

Succinic semialdehyde dehydrogenase deficiency (SSADHD) is a rare genetic disorder in the second enzyme of GABA degradation [8,9]. The phenotype encompasses nonspecific neurological features, including developmental delay, absence of formulated speech, hypotonia, and neuropsychiatric disturbances in adolescence and adulthood. The biochemical hallmark of SSADHD is accumulation of GHB in physiological fluids, including urine, plasma, and cerebrospinal fluid (CSF) [10] (Fig. 1). As well, GABA is elevated in CSF of SSADHD-patients for whom diagnostic lumbar puncture has been performed [11]. The non-specific features of this disorder, and the recent report of a man who was diagnosed with SSADHD not before the age of 63 years, suggests that SSADHD is underdiagnosed [12]. Accordingly, a rapid and high throughput assay to detect GHB in biofluids might be beneficial for screening for SSADHD in targeted populations.

In collaboration with the University of Applied Sciences and Arts of Northwestern Switzerland, Buhlmann Laboratories (Schoenenbuch, Switzerland) developed an enzymatic assay to determine GHB in serum and urine in 2011. The method was developed to detect the recreational use of GHB (e.g. intoxication), and it can be run on clinical chemistry analyzers which are generally available around the clock. This assay employs recombinant GHB dehydrogenase (GHBDH, EC 1.1.1.61), which catalyzes the oxidation of GHB to succinic semialdehyde with stoichiometric production of NADH which is quantitatively at 340 nm [13,14]. Here, we have evaluated the capacity of this enzymatic assay to identify patients with SSADHD via quantitation of GHB.

2. Materials and methods

2.1. Biological samples

Urine and plasma from patients with SSADHD and control individuals was obtained with informed consent. Patient age range was 2–37 years. Control individuals included unrelated individuals.

![Fig. 1. Metabolic pathway of glutamic acid in SSADH-deficient patients. The black bar indicates the deficient succinic semialdehyde dehydrogenase (SSADH) and the bold arrows show the metabolic pathway of glutamic acid in SSADH-deficient patients where GHB accumulates. Modified according to Gahr et al. [24] and Pearl et al. [25].](image-url)
Fig. 2. Comparison of the GC-FID method as reference method vs. the enzymatic method. A) Passing-Bablok-plot, first study (proof of concept) with urine N = 7 — indicates the slope 1.3213 ---- indicates the 95% CI [1.0371, 1.7892] B) Bland Altman plot. r = 0.997, p ≤ 0.001.

Fig. 3. Comparison of the GC-MS method as reference method vs. the enzymatic method. A) Passing-Bablok-plot, first study with urine N = 7 — indicates the slope 1.0008 ---- indicates the 95% CI [0.7884, 1.3241] B) Bland Altman plot (right). r = 0.991, p ≤ 0.001.
It remains to be determined if GHB in physiological conditions decreases/increases with age, thus necessitating age-matching with controls for comparison. Samples from both genders were included, as there is contradictory evidence about gender distinctions in GHB levels of physiological fluids [15–18]. For both studies all samples were split and sent to analytical laboratories for GHB measurements in anonymized fashion.

2.2. Enzymatic assay for GHB

GHB was quantified on a Cobas Integra 400 Plus [14]. Calibrators (lyophilized GHB in water, Lot 3815 10 mg/L and 100 mg/L) and controls (lyophilized GHB in human urine, Lot 3815: low control 12.6 mg/L and high control 68 mg/L GHB) were provided by Buhlmann laboratories AG and reconstituted with 2 ml distilled water for use. For enzymatic incubation, the buffer was Lot 3814, cofactor (NAD+ adjective) Lot 4615, and recombinant enzyme Lot 4616. The standard curve for NADH production was measured with two calibrators described above in duplicate, and was valid for 14 days. Although analytical sensitivity, imprecision, recoveries, and assay linearity have been previously reported [14], these parameters were re-evaluated in the current study.

In a screening setting the limit of detection (LOD) described as 1.5 mg/L (−14.4 μmol/L) can be used in a qualitative meaning to decide whether a sample is positive or negative for elevated GHB levels. The lower limit of quantification (LLOQ) for serum is stated as 4.5 mg/l (−43.2μmol/L) and for urine 2.8 mg/l (29.6 μmol/L) [14]. For qualitative evaluation in a screening setting the LOD can be used to decide if the sample is positive or negative for elevated GHB- levels. The LLOQ must be considered when quantitative results are needed or a ratio will be calculated (e.g. for urine- creatinine ratio).

Imprecision was assessed as within-run precision (intra-assay CV) using 10 repetitive measurements of the GHB low and high controls, respectively, and calculated using Excel (Version 2010, Microsoft, Redmond, USA). Between-daily imprecision (inter-assay-CV) was determined by measuring low and high controls twice a day for 5 days (N = 10). The dilution linearity was determined in the urine containing the highest GHB concentration. The urine sample was pre-diluted 1:2 with NaCl 0.9%. This solution was further diluted with NaCl 0.9% containing the highest GHB concentration. The urine sample was pre-diluted 1:2 with NaCl 0.9%. This solution was further diluted with NaCl 0.9% containing the highest GHB concentration.
Fig. 4. Comparison of the GC–MS IDM method as reference method vs. the enzymatic method. A) Passing-Bablok-plot, second study with urine N = 10. — indicates the slope 0.9173 ---- indicates the 95% CI [0.1596, 2.3876] B) Bland Altman plot. $r = 0.738$, $p \leq 0.01$.

Fig. 5. Comparison of the GC–MS as reference method vs. the enzymatic method. A) Passing-Bablok-plot, second study with urine N = 10. — indicates the slope 0.9864 ---- indicates the 95% CI [0.9093, 1.2854] B) Bland Altman plot. $r = 0.996$, $p \leq 0.001$. 
differences in the measurements occur in patients with higher GHB levels.

3.3. Plasma GHB

For plasma, we applied a cutoff level of 10 mg/L (−96.1 μmol/L) for GHB, which was the value employed by Hasan and coworkers (2011), although that value was derived for sera. Using our cutoff level for GHB in plasma revealed that all 14 control specimens would have been considered negative for GHB concentration. However, 6 of 17 patient samples would have been identified as negative for elevated GHB (e.g., false negatives). Using the cutoff level for serum determined with the GC-MS method of 4 mg/mL (~38.4 μmol/L) published by Andresen and coworkers (2010), all control samples except one would have been considered negative for GHB concentration (e.g., 1 false positive). In Table 5 the GHB concentrations of all plasma samples are summarized and consequences of the cutoff setting are displayed. The comparison of the different methods is shown in Figs. 6–9. Overall, the enzymatic assay displayed higher values in plasma for all samples measured (Fig. 9).

| Enzymatic method | GC-MS IDMS GHB (μmol/L) | GC-MS GHB (μmol/L) | Group | Cutoff | Cutoff |
|------------------|-------------------------|--------------------|-------|--------|--------|
|                  |                         |                    |       | 38.4 μmol/L (4 mg/L) | 96.1 μmol/L (10 mg/L) |
| <14.4            | 1.2                     | <5.8               | Control | Negative | Negative |
| <14.4            | 0.9                     | <5.8               | Control | Negative | Negative |
| <14.4            | <5.8                    | <5.8               | Control | Negative | Negative |
| <14.4            | 1.3                     | <5.8               | Control | Negative | Negative |
| <14.4            | 1.3                     | <5.8               | Control | Negative | Negative |
| <14.4            | 2.2                     | ~5.8               | Control | Negative | Negative |
| <14.4            | 0.7                     | <5.8               | Control | Negative | Negative |
| 16.3             | 1.9                     | <5.8               | Control | Negative | Negative |
| 18.3             | 2.9                     | 6.9                | Control | Negative | Negative |
| 18.3             | 0.6                     | <5.8               | Control | Negative | Negative |
| 21.1             | 0.8                     | <5.8               | Control | Negative | Negative |
| 21.1             | <5.8                    | <5.8               | Control | Negative | Negative |
| 27.9             | 0.7                     | <5.8               | Control | Negative | Negative |
| 42.3             | 36.2                    | 37.2               | Patient | Positive | Negative |
| 42.3             | 1.1                     | <5.8               | Control | Negative | Negative |
| 46.1             | 35.2                    | 38.9               | Patient | Positive | Negative |
| 60.5             | 48.9                    | 44.0               | Patient | Positive | Negative |
| 71.1             | 29.9                    | 26.0               | Patient | Positive | Negative |
| 75.9             | 25.5                    | 28.6               | Patient | Positive | Negative |
| 79.7             | 59.6                    | 47.2               | Patient | Positive | Negative |
| 80.8             | 42.2                    | 35.6               | Patient | Positive | Positive |
| 89.9             | 67.6                    | 62.0               | Patient | Positive | Positive |
| 122.0            | 69.6                    | 61.6               | Patient | Positive | Positive |
| 210.4            | 168.0                   | 137.6              | Patient | Positive | Positive |
| 264.2            | 111.0                   | 113.5              | Patient | Positive | Positive |
| 358.3            | 298.0                   | 222.7              | Patient | Positive | Positive |
| 363.1            | 277.0                   | 236.9              | Patient | Positive | Positive |
| 468.8            | 373.0                   | 278.8              | Patient | Positive | Positive |
| 604.2            | 497.0                   | 400.1              | Patient | Positive | Positive |
| 663.8            | 533.0                   | 430.0              | Patient | Positive | Positive |
| 689.7            | 523.0                   | 440.2              | Patient | Positive | Positive |

In conclusion, we demonstrated that an established method for the enzymatic determination of GHB concentrations run on a routine clinical chemistry analyzer using urine. The method is rapid, robust and inexpensive. The assay appears to be sufficient sensitive and reliable, and could be used for the identification of SSADHD in the frame of a screening scheme with subsequently confirmation through chromatographic analysis and genetic testing. For plasma we recommend a cutoff concentration for 4 mg/L (~38.4 μmol/L) and for urine a urine creatinine-normalized GHB concentration of 5 mmol/mol creatinine.

Table 5
GHB plasma concentrations in controls and patients from the second study using 3 different analytical methods. Influence of cutoff setting on false positive and false negative. 1 control (bold) assessed as false positive when cutoff set at 38.4 μmol/L, whereas 6 patients assessed as false negative, when cutoff set at 96.1 μmol/L.
Fig. 6. Comparison of the GC–MS method IDM as reference method vs. the enzymatic method. A) Passing-Bablok-plot, second study with plasma $N = 17$ — indicates the slope 1.2399 ---- indicates the 95% CI [1.1600, 1.3313] B) Bland Altman plot. $r = 0.999$, $p \leq 0.001$.

Fig. 7. Comparison of the GC–MS method as reference method vs. the enzymatic method. A) Passing-Bablok-plot, second study with plasma $N = 17$. — indicates the slope 1.5656 ---- indicates the 95% CI [1.4668, 1.7403] B) Bland Altman plot. $r = 0.993$, $p \leq 0.001$. 
Fig. 8. Urine creatinine normalized GHB (upper graphs) and plasma sample (lower graphs) derived from controls and patients. The x-axis indicates the method used. Abbreviations: GC-MS, gas chromatography–mass spectrometry, GC-MS IDM, gas chromatography–mass spectrometry with isotope dilution.

Fig. 9. GHB concentration in plasma samples derived from controls and patients. The abbreviations and descriptions are identical to those of Fig. 8.
Conflict of interest

All authors declare that they have no conflict of interest. All samples evaluated in this study were obtained with informed consent (Washington State University, IRB #12678).

Acknowledgements

The authors acknowledge with gratitude the patients and family members who contributed samples to this study. The support of the SSADH Association (www.ssadh.net) and Speragen, Inc., is also gratefully acknowledged. This study is supported in part by NIH R21 NS 85369 and R01 NS 82286.

References

[1] N.G. Bowery, T.G. Smart, GABA and glycine as neurotransmitters: a brief history, Br. J. Pharmacol. 147 (Suppl. 1) (2006) S109–S119.

[2] H.S. Waagepetersen, U. Sonnewald, A. Schousboe, The GABA paradox: multiple roles as metabolite, neurotransmitter, and neurodifferentiative agent, J. Neurochem. 73 (1999) 1335–1342.

[3] M. Maitre, C. Klein, A.G. Mensah-Nyagan, Mechanisms for the specific properties of gamma-hydroxybutyrate in brain, Med. Res. Rev. 36 (2016) 363–388.

[4] M. Maitre, The gamma-hydroxybutyrate signalling system in brain: organization and functional implications, Prog. Neurobiol. 51 (1999) 337–361.

[5] O.C. Snead, K.M. Gibson, Gamma-Hydroxybutyric Acid New England, J. Med. 352 (2005) 2721–2732.

[6] C.G. Wong, K.M. Gibson, O.C. Snead 3rd, From the street to the brain: neurobiology of the recreational drug gamma-hydroxybutyric acid, Trends Pharmacol. Sci. 25 (2004) 29–34.

[7] F.P. Busardo, A.W. Jones, GHB pharmacology and toxicology: acute intoxication, concentrations in blood and urine in forensic cases and treatment of the withdrawal syndrome, Curr. Neuropharmacol. 13 (2015) 47–70.

[8] M. Parviz, K. Vogel, K.M. Gibson, P.L. Pearl, Disorders of GABA metabolism: SSADH and GABA-transaminase deficiencies Journal of pediatric epilepsy 3 (2014) 217–227.

[9] K.K. Vogel, P.L. Pearl, W.H. Theodore, R.C. McCarter, C. Jakobs, K.M. Gibson, Thirty years beyond discovery—clinical trials in succinic semialdehyde dehydrogenase deficiency, a disorder of GABA metabolism. J. Inherit. Metab. Dis. 36 (2013) 401–410.

[10] K.M. Gibson, C. Jakobs, H. Ogier, L. Hagenfeldt, K.E. Eeg-Olofsson, D. Eeg-Olofsson, F. Aksu, H.P. Weber, E. Rossier, B. Vollmer, et al., Vigabatrin therapy in six patients with succinic semialdehyde dehydrogenase deficiency. J. Inherit. Metab. Dis. 18 (1995) 143–146.

[11] K.M. Gibson, M. Gupta, P.L. Pearl, M. Tuchman, L.G. Vezina, O.C. Snead, L.M.E. Smit, C. Jakobs, Significant behavioral disturbances in succinic semialdehyde dehydrogenase (SSADH) deficiency (Gamma-Hydroxybutyric aciduria). Biol. Psychiatry 54 (2003) 763–768.

[12] S. Lapalme-Remis, E.C. Lewis, C. De Meuleneire, P. Chakraborty, K.M. Gibson, C. Torres, A. Guberian, G.S. Salomons, C. Jakobs, A. Ali-Ridha, M. Parviz, P.L. Pearl, Natural history of succinic semialdehyde dehydrogenase deficiency through adulthood, Neurology 85 (2015) 861–865.

[13] M.A. Scio, L. Hasan, A. Scholer, T.M. Jermann, J.M. Weber, D. Gygax, Development and characterization of an enzymatic method for the rapid determination of gamma-hydroxybutyric acid, Chim. 64 (2010) 793–798.

[14] L. Hasan, T.M. Jermann, J.M. Weber, L. Abrahamsson, M.A. Scio, M. Bottcher, W. Jochle, D. Gygax, A. Scholer, An enzymatic method to determine gamma-hydroxybutyric acid in serum and urine, Ther. Drug Monit. 33 (2011) 757–765.

[15] P. Kintz, V. Cirimele, C. Jamey, B. Lukes, Testing for GHB in hair by GC/MS/MS after a single exposure. Application to document sexual assault, J. Forensic Sci. 48 (2003) 195–200.

[16] D.T. Yeatman, K. Reid, A study of urinary endogenous gamma-hydroxybutyrate (ggh) levels, J. Anal. Toxicol. 27 (2003) 40–42.

[17] A.L. Castro, M. Dias, F. Reis, H.M. Teixeira, Gamma-hydroxybutyric acid endogenous production and post-mortem behaviour – the importance of different biological matrices, cut-off reference values, sample collection and storage conditions, J. Forensic Leg. Med. 27 (2014) 17–24.

[18] F. Valaio, G. Serpelloni, S. Furlanetto, D. Palumbo, F. Mari, A. Fioravanti, E. Bertol, Determination of endogenous concentration of g-hydroxybutyric acid (GHB) in hair through an ad hoc GC–MS analysis: A study on a wide population and influence of gender and age, J. Pharm. Biomed. Anal. 118 (2016) 161–166.

[19] M.A. Lebeau, M.A. Montgomery, M.L. Miller, S.G. Burmeister, Analysis of biomarkers for gamma-hydroxybutyrate (GHB) and gamma-butyrolactone (GBL) by headspace GC-FID and GC–MS analysis: A study on a wide population and influence of gender and age, J. Pharm. Biomed. Anal. 118 (2016) 161–166.

[20] H. Andresen, N. Sprys, A. Schmidt, A. Mueller, S. Iwersen-Bergmann, Gamma-hydroxybutyrate in urine and serum: additional data supporting current cut-off recommendations, Forensic Sci. Int. 200 (2010) 93–99.

[21] K.M. Gibson, S. Aramaki, L. Veenman, W.L. Nyhan, D.C. DeVivo, A.K. Hodson, C. Wernli et al. / Molecular Genetics and Metabolism Reports 11 (2017) 81–89