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

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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 alone (proof of concept) 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: $r = 0.997$, $p \le 0.001$; GC–MS: $r = 0.99$, $p \le 0.001$); 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; $r = 0.993$, $p \le 0.001$).
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 the 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.

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); GHBDH, GHB-dehydrogenase; IDM, isotope dilution method; LOD, limit of detection; LLOQ, lower limit of quantification; NADH, nicotinamide adenine dinucleotide, reduced form; r, correlation coefficient (Pearson); SSADHD, succinic semialdehyde dehydrogenase deficiency.

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[3] Salomons GS, Jansen EE-additional laboratory analyses, manuscript preparation, editing.
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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 quantified spectrophotometrically at 340 nm [13,14]. Here, we have evaluated the capacity of this enzymatic assay to identify patients with SSADHD via quantitation of GHB.

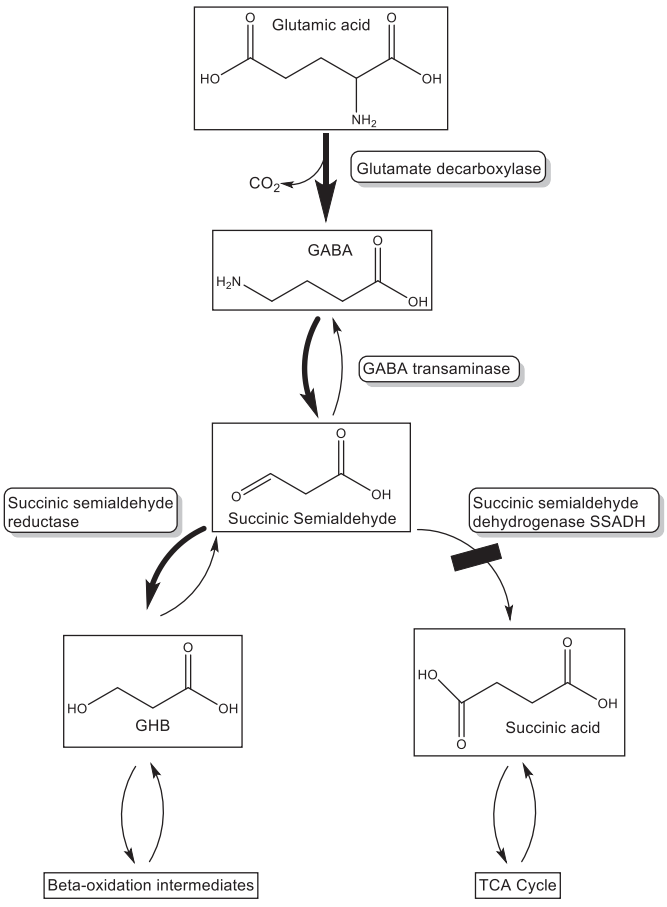

Fig. 1. Metabolic pathway of glutamic acid in SSADH-deficient patients. The black bar indicates the deficient succinic semialdehyde dehydrogenase (SSADH) and the bold arrows show the metabolic pathway of glutamic acid in SSADH-deficient patients where GHB accumulates. Modified according to Gahr et al. [24] and Pearl et al. [25].

Table 1
Imprecision of the enzymatic assay (Cobas Integra 400 plus), concentrations of the controls were: low control 12.6 mg/L, high control 68.0 mg/L.
Abbreviation: CV, coefficient of variation.

Imprecision	Low control (%)	High control (%)
Intra-assay CV (N = 10)	4.2	1.1
Inter-assay CV (N = 10)	6.8	4.1

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

Table 2
Dilution linearity of the enzymatic assay on a Cobas Integra 400 plus device, determined by dilution of the urine sample from patient No. 3 with NaCl 0.9%.

Dilution of urine sample no. 3	Calculated GHB-concentration (mg/L)	Measured GHB-concentration (mg/L)	Recovery (%)
Prediluted 1:2	–	180.8	n/a
1:2	90.4	96.6	107
1:5	36.2	42.4	117
1:10	18.1	21.3	118
1:20	9.0	9.4	104
1:50	3.6	2.9	81
Mean recovery	–	–	105.4

Table 3
Detailed results of urine creatinine-normalized GHB-concentrations in all 7 SSADHD patients in urine from the first study.
Abbreviation: GC–MS, gas chromatography–mass spectrometry, GC-FID, gas chromatography- flame ionization detector.

Sample no.	Enzymatic method GHB (mmol/mol creatinine)	GC-FID GHB (mmol/mol creatinine)	GC–MS GHB (mmol/mol creatinine)
1	21.30	21.1	16.3
2	79.25	55.5	54.2
3	92.40	62.2	77.6
4	162.20	142.2	149.3
5	315.40	277.2	238.3
6	365.47	214.8	417.2
7	681.09	511.0	665.8

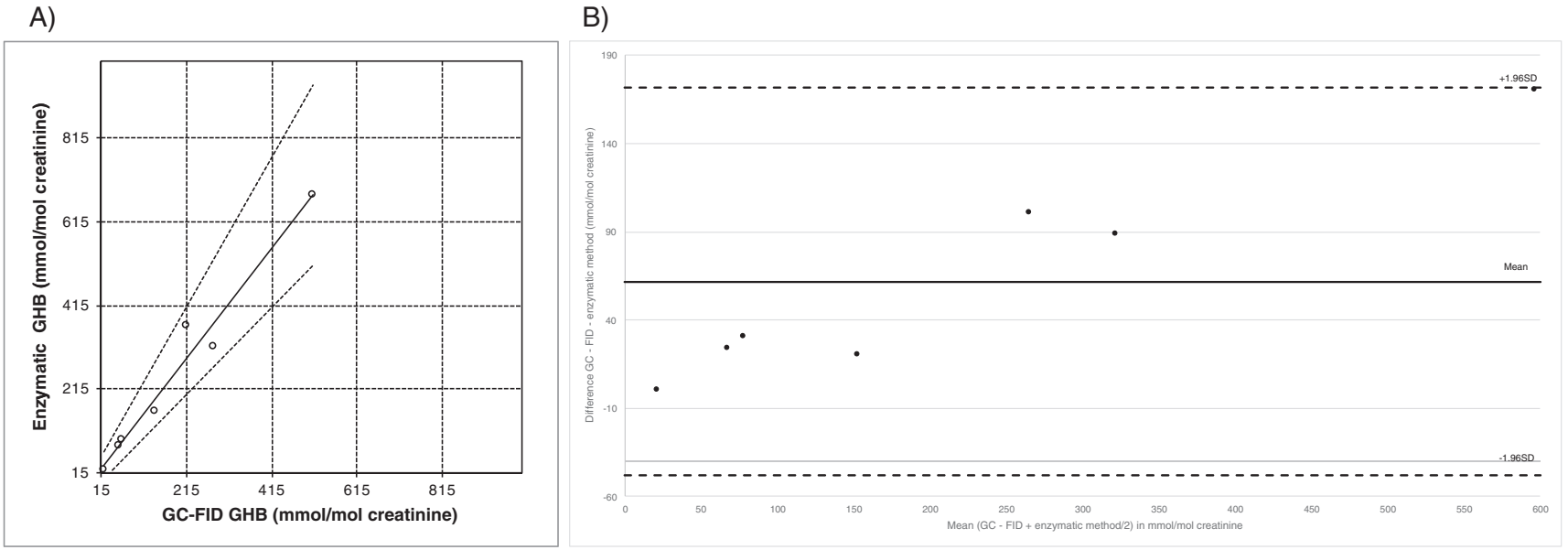

Fig. 2. Comparison of the GC-FID method as reference method vs. the enzymatic method. A) Passing-Bablok-plot, first study (proof of concept) with urine N= 7 — indicates the slope 1.3213 ---- indicates the 95% CI [1.0371, 1.7892] B) Bland Altman plot. r = 0.997, p ≤0.001.

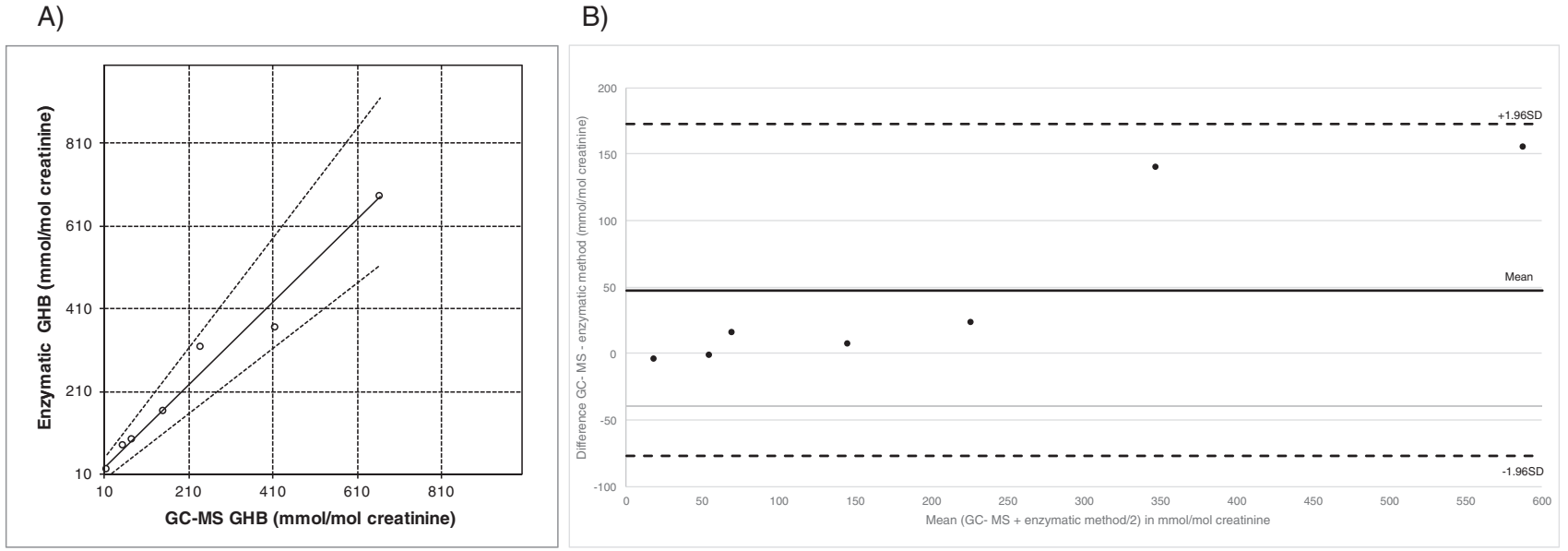

Fig. 3. Comparison of the GC–MS method as reference method vs. the enzymatic method. A) Passing-Bablok- plot, first study with urine N = 7 — indicates the slope 1.0008 ---- indicates the 95% CI [0.7884, 1.3248] B) Bland Altman plot (right). r = 0.991, p ≤0.001.

(adults) as well as parents/siblings of patients (obligate heterozygotes since SSADHD is an autosomal-recessively inherited disorder). It remains to be determined if GHB in physiological fluids 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 deionized water for use. For enzymatic incubation, the buffer was Lot 3814, cofactor (NAD^+) 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 (~43.2 μmol/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% to 1:2, 1:5, 1:10, 1:20, and 1:50. Urine concentration of GHB was corrected to creatinine content using the creatinine plus version 2 test on the Cobas 400 plus from Roche Diagnostics (Rotkreuz, Switzerland). Urine creatinine controls were obtained from Roche Diagnostics, Rotkreuz, Switzerland.

2.3. Comparator assay methods and statistical analyses

Aliquots of each sample analyzed with the enzymatic assay were also measured with three chromatographic methods: GC-FID (proof of concept study) [19] and two GC–MS methods after extraction and derivatization of GHB. One is mentioned as “GC–MS” (used for the proof of concept and the feasibility study as [20]), and the other method is mentioned as “GC-MS IDM” (only for feasibility study [21])

Statistical analysis of the data was performed with Excel (Version 2015, Redmond, USA) employing the Passing-Bablok and Bland-Altman plots using the Acomed Statistics Template for Excel (Version 3.0, Leipzig, Germany) and OriginPro (Version 2016, Northampton, USA).

3. Results

3.1. Precision and accuracy

The manufacturer specifies the precision of the enzymatic assay conducted on a KoneLab 30 (Thermo Fisher Scientific Inc., Waltham, USA) as intra-assay CV <10% and inter-assay <5% CV, total precision <10%. Dilution linearity is specified in urine as 100–105% (mean 103%). We re-evaluated these parameters on our instrumentation, which was different. We found a higher inter-CV of the low control, but the total CV was below 10%. The dilution linearity for the urine sample containing the highest GHB concentration in our hands was determined from 81 to 118%, with a mean recovery of 105.4%, and the results are displayed in Tables 1 and 2. Overall, the assay appeared to respond as expected, and we proceeded to blinded samples of urine and plasma.

3.2. Urine GHB

GHB concentrations in urine derived from patients are displayed in Table 3. This table shows data from the initial proof of concept study determined by the enzymatic assay, by GC-FID method and by GC–MS respectively. In this study, urine derived from five control individuals showed a level of GHB lower than then LOD (<1.5 mg/L; ~<14.4 μmol/L). These results for negative controls were comparable to the manufacturer's data. The urine samples derived from seven patients with SSADHD revealed elevated GHB levels that correlated well with GC-FID and GC–MS. Ranges for GHB level in patient urine (n = 7) were: enzymatic assay, 21–681 mmol/mol creatinine; GC-FID, 21–511 mmol/mol creatinine; GC–MS, 16–666 mmol/mol creatinine. The graphs in which the three methods were compared are shown in Figs. 2 and 3. Larger differences between the enzymatic method and chromatographic methods were observed in patients with higher GHB levels. Based on these results, we initiated the feasibility study that encompassed a much larger patient cohort, as well as plasma samples.

In the feasibility study, the level of GHB for 23 control individuals was generally <5 mmol/mol creatinine (not displayed in the table), consistent with previously published values for normal individuals [21]. In two samples, a low creatinine (1 and 1.9 mmol/L) was likely responsible for the higher values of GHB obtained (7.6–19.6 mmol/mol creatinine).

Moreover, the absolute amounts of GHB in these two samples were below the lower limit of quantification for the enzymatic assay, and thus would be reported as non-detectable. Ranges of GHB concentrations for patient and control samples (with omission of the two low creatinine specimens) were: enzymatic assay, patient 17–345 mmol/mol creatinine (concentration in control samples <4.5 mmol/mol creatinine); GC–MS, 34–514 mmol/mol creatinine (concentrations in control samples <4.1 mmol/mol creatinine); and GC–MS, 13–350 mmol/mol creatinine (concentration in control samples <2.5 mmol/mol creatinine). Detailed results of GHB values for all patients can be seen in Table 4. The method comparison is plotted in Figs. 4, 5, and 8. As already observed in the first study,

Table 4
Detailed results of urine creatinine-normalized GHB-concentrations in all 10 SSADHD patients in urine from the second study. The abbreviations and descriptions are identical to those of Table 1, GC–MS IDM, gas chromatography-mass spectrometry with isotope dilution.

Sample no.	Enzymatic method GHB (mmol/mol creatinine)	GC–MS IDM GHB (mmol/mol creatinine)	GC–MS GHB (mmol/mol creatinine)
1	16.6	69.1	12.8
2	26.5	200.3	26.6
3	30.9	34.0	26.2
4	33.5	175.0	25.0
5	51.8	199.6	45.3
6	71.7	221.1	61.8
7	76.4	83.6	59.0
8	207.2	223.0	242.3
9	242.1	153.0	254.4
10	345.0	513.8	349.5

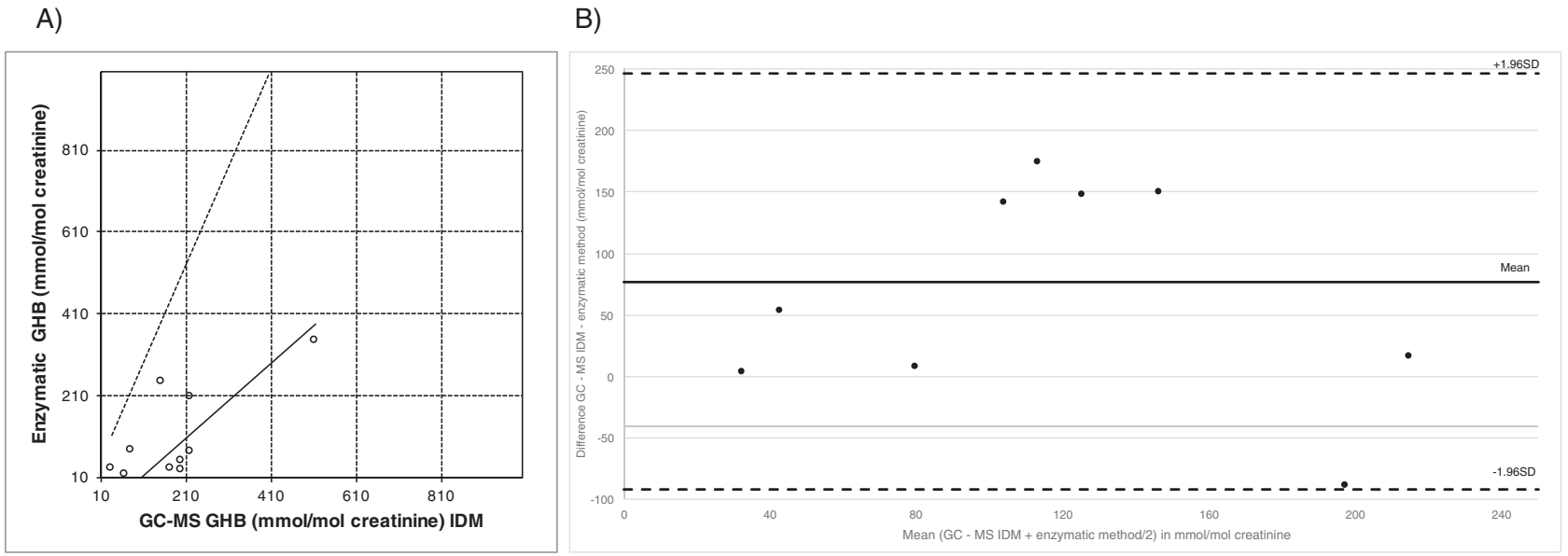

Fig. 4. Comparison of the GC–MS IDM method as reference method vs. the enzymatic method. A) Passing-Bablok- plot, second study with urine N = 10. — indicates the slope 0.9173 ---- indicates the 95% CI [0.1596, 2.3876] B) Bland Altman plot. r = 0.738, p ≤0.01.

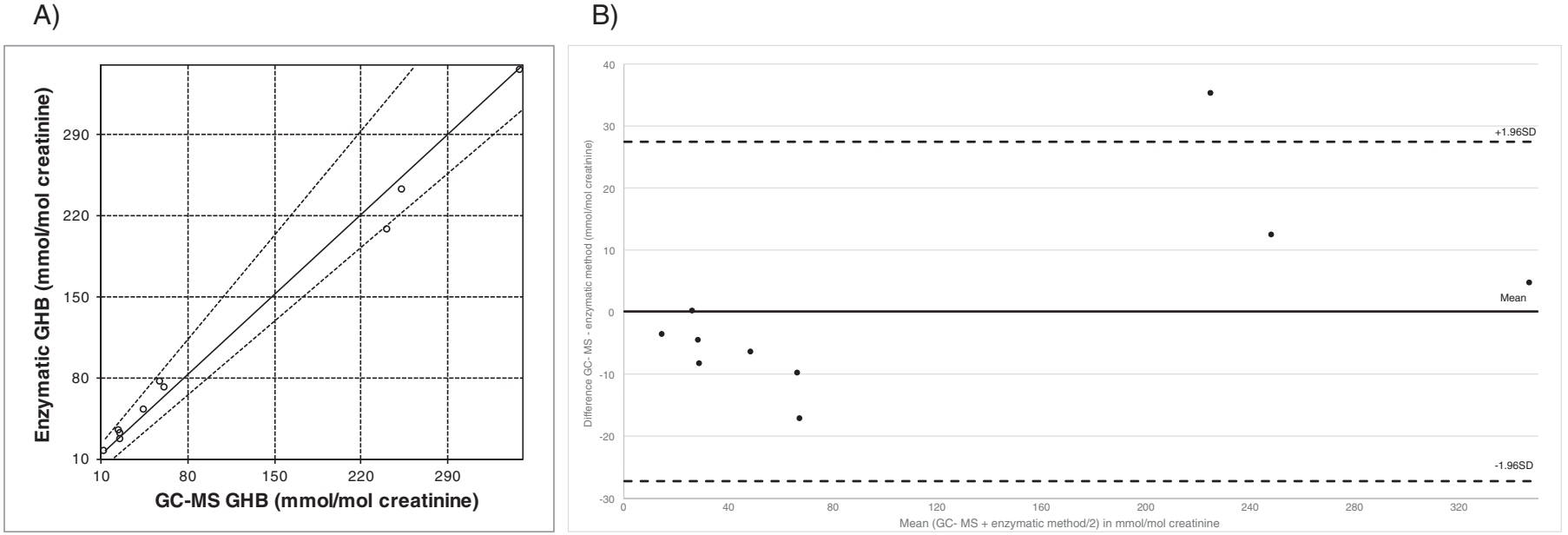

Fig. 5. Comparison of the GC–MS as reference method vs. the enzymatic method. A) Passing-Bablok- plot, second study with urine N = 10. — indicates the slope 0.9864 ---- indicates the 95% CI [0.9093, 1.2854] B) Bland Altman plot. r = 0.996, p ≤0.001.

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. The abbreviations and descriptions are identical to those of Table 2.

Enzymatic method GHB (μmol/L)	GC–MS IDM GHB (μmol/L)	GC–MS GHB (μmol/L)	Group	Cutoff 38.4 μmol/L (4 mg/L)	Cutoff 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	**Positive**	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
98.0	42.2	35.6	Patient	Positive	Positive
99.9	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

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 specimens 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).

4. Discussion

Our results suggest that the enzymatic assay for GHB detection is sufficiently robust to screen for SSADHD in selected populations employing urine, but not plasma. This may not pose a major challenge in terms of screening, since collection of urine will be less challenging than venipuncture for blood collection and plasma isolation. Moreover, as a screening assay for SSADHD, the potential to obtain an additional sample for a "suspicious" patient (whose GHB level may be at the lower limit of detection, but whose clinical picture might be suggestive) is realistic (whether urine or blood). However, when using the assay in the context of a drug screen, or that of acquaintance sexual assault, the possibility to obtain a second sample is limited, and the assay system must provide the requisite sensitivity and specificity for accurate detection.

Generally, the enzymatic assay yielded higher values for GHB in urine compared to the gas chromatography methods. For urine samples derived from patients with SSADHD, the enzymatic assay correlated well with the GC–MS method. For the IDM method, there was excellent agreement between all three laboratories for 3 samples, a moderate discrepancy between 3 samples, and fairly significant differences for 4 samples. Nevertheless, by omitting analysis of the two urine samples with low creatinine, all labs would have accurately identified patients with elevated GHB and thus increased suspicion for SSADHD.

The identification of SSADHD would be very dependent upon where the GHB cutoff for the enzymatic assay was set, which was especially true for plasma. For example, by setting the cutoff concentration for GHB of 4 mg/L (~38.4 μmol/L) [20] for serum one control plasma sample would have been considered positive (false positive). Conversely, by setting the cutoff concentration for GHB at 10 mg/L (~96.1 μmol/L) as recommended by the manufacturer, 6 out of 17 patient samples would have been considered negative (false negatives). When the GHB concentration in plasma samples were higher (generally >100 μmol/L), the enzymatic analysis was robust (Fig. 9). Using a cutoff level for urine creatinine- normalized GHB- concentrations of 5 mmol/mol creatinine, the enzymatic assay was accurate and sensitive in urine. Further analysis using GC–MS techniques followed by genetic testing to confirm or exclude SSADHD as the cause of elevated GHB could be pursued as well. Importantly, according to the manufacturer of the enzymatic assay, serum should be used to determine GHB levels in blood, which may explain the higher levels detected in plasma employing the enzymatic assay.

SSADHD is not currently on the expanded list of newborn screening disorders, although a method for quantitation of GHB in dried bloodspots has been presented [22]. That method employed UPLC separation and liquid chromatography tandem mass spectrometry for quantitation of GHB is not easily applicable to newborn screening platforms. Conversely, an enzymatic assay run on a routine clinical chemistry analyzer may well be amenable to newborn screening, since it could be high-throughput with rapid turnaround. There would be precedent for such an application, since screening for biotinidase deficiency is performed on a secondary platform employing an enzymatic assay [23].

Further the enzymatic assay could be useful in basic research, where small volumes of samples are available (e.g. mouse blood). As well there might be an application in monitoring GHB levels during clinical studies with new drug candidates. It is noteworthy that the method can be ran on a routine clinical chemistry analyzer for example in the study center. There is no need to have access to special equipment and specialized technicians.

5. Conclusions

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 of 4 mg/L (~38.4 μmol/L and for urine a urine creatinine- normalized GHB concentration of 5 mmol/mol creatinine.

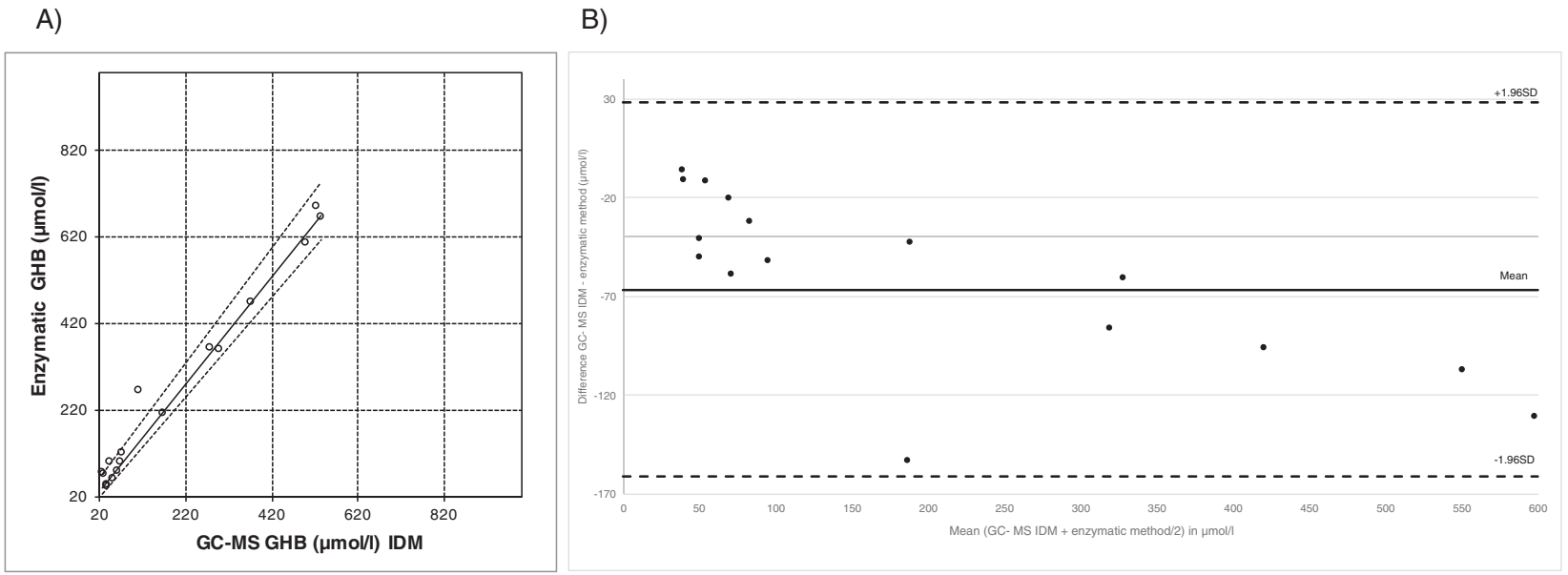

Fig. 6. Comparison of the GC–MS method IDM as reference method vs. the enzymatic method. A) Passing-Bablok- plot, second study with plasma $N = 17$ — indicates the slope 1.2399 ---- indicates the 95% CI [1.1600, 1.3313] B) Bland Altman plot. r = 0.999, p ≤0.001.

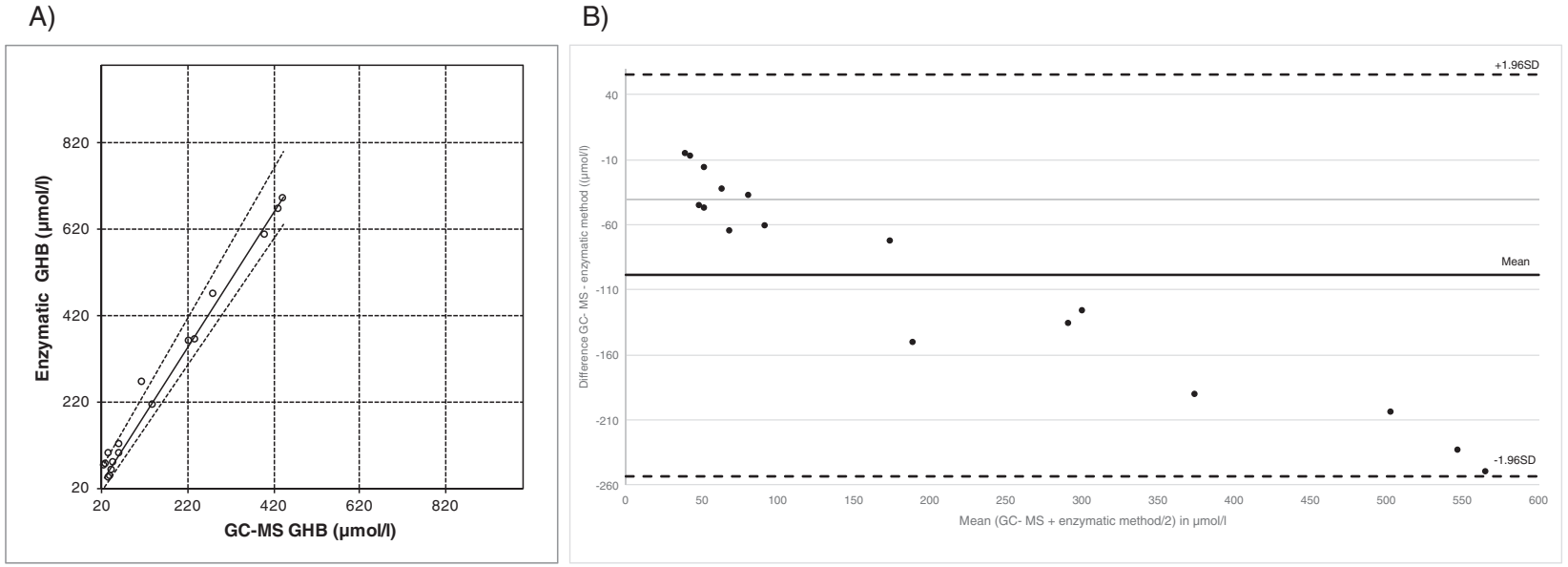

Fig. 7. Comparison of the GC–MS method as reference method vs. the enzymatic method. A) Passing-Bablok-plot, second study with plasma N = 17. — indicates the slope 1.5656 ---- indicates the 95% CI [1.4668, 1.7403] B) Bland Altman plot. r = 0.993, p ≤0.001.

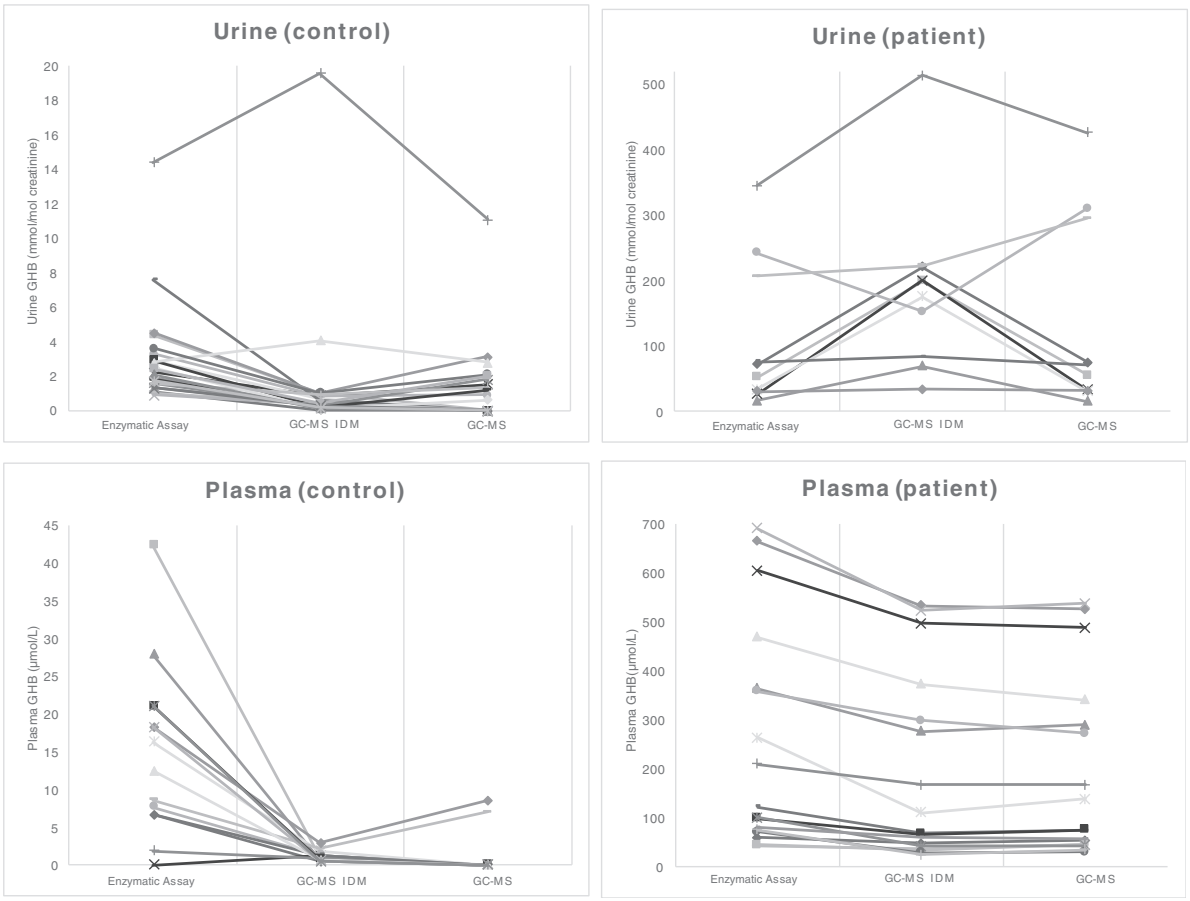

Fig. 8. Urine creatinine normalized GHB (upper graphs) and plasma sample (lower graphs) derived from controls and patients. The x-axis indicates the method used. Abbreviations: GCMS, gas chromatography–mass spectrometry, GC- MS IDM, gas chromatography–mass spectrometry with isotope dilution.

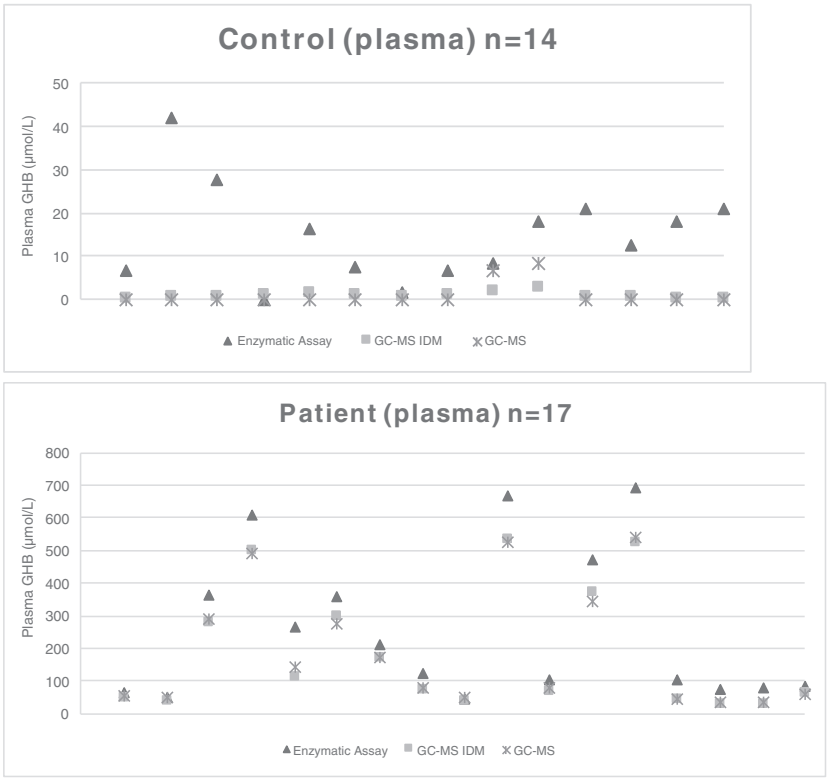

Fig. 9. GHB concentration in plasma samples derived from controls and patients. The abbreviations and descriptions are identical to those of Fig. 8.

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).

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