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ORIGINAL ARTICLE

Endogenous gamma-hydroxybutyric acid (GHB) concentrations in post-mortem specimens and further recommendation for interpretative cut-offs

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Abstract When interpreting gamma-hydroxybutyric acid (GHB) concentrations in post-mortem specimens, a possible increase in GHB concentrations because of post-mortem generation must be considered. In this study, endogenous GHB concentrations in post-mortem biological fluids were investigated. Additionally, we review post-mortem GHB concentrations already published in the literature. Heart and peripheral blood samples, cerebrospinal fluid, urine, and vitreous humor were collected from 64 autopsies in subjects where the cause of death excluded GHB exposure. Sample analysis was carried out either on the day of autopsy or later after immediate freezing and storage at −20 °C. GHB concentrations in venous blood samples (n=61) were <0.6–28.7 mg/L (mean 11.9 mg/L; median 10.6 mg/L), <0.6–65.3 mg/L (mean 15.2 mg/L; median 12.8 mg/L) in heart blood (n=56), <0.6–25.1 mg/L (mean 6.0 mg/L; median 3.8 mg/L) in urine (n=50), <0.6–39.0 mg/L (mean 9.6 mg/L; median 7.5 mg/L), in vitreous humor (n=54), and <0.6–24.0 mg/L (mean 4.2 mg/L; median 3.2 mg/L) in cerebrospinal fluid (n=52). There was no significant difference between GHB concentrations in cases where there were signs of beginning putrefaction at the time of autopsy (n=9) and cases without obvious signs of putrefaction. In one case with advanced putrefaction, the GHB concentration in venous blood was 32.7 mg/L. In conclusion, for post-mortem venous blood, urine, and cerebrospinal fluid, an interpretative cut-off of 30 mg/L for GHB concentrations is suggested in cases where GHB analysis is conducted on the day of sample collection at autopsy or if samples have been stored at −20 °C immediately after collection.

Keywords Vitreous humor · Cerebrospinal fluid · Urine · Blood · Putrefaction

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Introduction

Gamma-hydroxybutyric acid (GHB) is used in several countries for medical purposes as an anesthetic adjuvant and for the treatment of narcolepsy with cataplexy. Additionally, GHB is commonly used as a recreational drug with an initial stimulant-like effect and a subsequent sedative effect. Because of the high potential for abuse, GHB addiction and withdrawal can occur [1–3].

Because GHB is subject to the Narcotics Act, legal substitutes, such as gamma-butyrolactone (GBL) or 1,4-butanediol (1,4-BD), are used instead. Both of these are pro-drugs and are rapidly metabolized in vivo into the active compound GHB.

Several authors have reported clinically relevant intoxications by GHB or its substitutes, leading to bradycardia, seizures, respiratory depression, and impaired consciousness to the point of severe coma [4–7]. Fatalities have been reported with accidental overdoses of GHB [8–12], as well as suicidal intent, or trauma as a result of impaired driving [13, 14]. Fatal overdoses of GHB also occur with the precursors gamma-butyrolactone [15] or 1,4-butanediol [14, 16]. Moreover, GHB is a substance that is frequently detected in cases of drug-facilitated sexual assaults [17–19].

GHB is not only used as a drug, but is also present as a physiological transmitter in all human tissues and fluids [20, 21]. Therefore, in cases of positive GHB detection, there needs to be discrimination between endogenous and exogenous concentrations. In cases of clinical intoxication, GHB concentrations are higher than endogenous levels, depending on the time of sampling. However, in drug-facilitated sexual assault cases, the time interval between the assumed drug admission and collection of urine and/or blood samples is often several hours. Because of the short half-life of GHB, GHB concentrations in these samples are low. With specimens from living people, many studies have been carried out leading to cut-off recommendations for GHB. For blood (serum/plasma) samples, 4 mg/L [22] and 5 mg/L [23, 24] as threshold GHB concentrations for discriminating between exogenous and endogenous GHB have been suggested. For urine, cut-offs for GHB concentrations of between 2 mg/L [25] and 10 mg/L [22–24] have been proposed. Recently, we recommended GHB concentrations of 4 and 6 mg/L as cut-offs for ante-mortem serum and urine samples, respectively [26].

In the field of post-mortem toxicology, there has not been sufficient collection of data to generate comparable cut-off recommendations for GHB. As shown in several studies, endogenous post-mortem GHB concentrations can be considerably higher than in ante-mortem samples [5, 27–29], although the mechanism of post-mortem generation of GHB concentrations has not yet been clarified.

Several possible explanations for post-mortem generation of GHB concentrations can be found in the literature. Some microorganisms are suspected of degrading the neurotransmitter gamma-aminobutyric acid (GABA) to GHB [22]. Moriya and Hashimoto suggested that glycolysis by bacteria may enhance endogenous GHB production [30]. Other authors have attributed post-mortem increases in GHB and GABA to decreased activity or cessation of the Krebs cycle and enzymatic changes that occur after death. GHB may be reduced from GABA via succinic semialdehyde by succinic semialdehyde-dehydrogenase [20, 31]. Snead et al. observed that GABA could not be the sole source for GHB and suggested 1,4-butanediol as another precursor of GHB in the brain [32]. However, Sakurada et al. were unable to detect increased succinic semialdehyde or 1,4-butanediol levels in post-mortem liver samples [33].

GHB is a metabolite which might be produced during the putrefaction process. The main substance involved in GHB generation appears to be putrescine (butane-1,4-diamine), which occurs in all human cells and plays a role in cell growth. This substance can be converted by enzymes to GABA and subsequently to GHB. Because GHB is not the end product of this metabolic pathway, a longer post-mortem interval, and thus a higher degree of decomposition, will not necessarily lead to higher GHB concentrations [34, 35]. A recent study showed that GHB undergoes glucuronidation during metabolism [36]. Concentrations of up to 5 mg/L GHB-glucuronide have been detected in human urine samples and could be the cause of elevated endogenous ante-mortem GHB levels [36].

In post-mortem peripheral blood samples, high concentrations of GHB up to 197 mg/L [29] have been reported, even in cases without GHB ingestion. Some authors have recommended preliminary cut-offs of GHB concentrations for post-mortem specimens, such as Moriya and Hashimoto, who proposed a limit of 10 mg/L for urine and 30 mg/L for blood in decedents showing little or no putrefaction (post-mortem interval [PMI] <48 h) [37]. This threshold was later applied by two retrospective Swedish studies [12, 14]. Kintz et al. [28] proposed a cut-off of 50 mg/L for GHB concentrations in post-mortem heart blood samples. They concluded that post-mortem heart blood GHB concentrations >50 mg/L are not sufficient to determine drug exposure and that it is essential to additionally analyze other biological fluids, including peripheral blood and vitreous humor using the same cut-off value. Analysis of urine in addition to blood samples because of lower GHB concentrations owing to less post-mortem generation has also been recommended [27, 38, 39]. Other authors have suggested that the vitreous humor is the specimen of choice in addition to femoral vein blood [28, 35]. Moriya and Hashimoto postulated that the proposed cut-off level of urine GHB values (10 mg/L) may also be applicable to analytical results in cerebrospinal fluid and vitreous humor [37]. However, there are still various interpretative problems for toxicologists because existing data are from different publications, which are difficult to compare.

This study was carried out to propose a scientific basis for discriminating between endogenous formation and GHB exposure, and to obtain reliable data regarding endogenous post-mortem GHB levels after optimal routine specimen handling (storage at −20 °C immediately after autopsy). Moreover, we compared GHB concentrations in several biological fluids (peripheral blood, heart blood, urine, cerebrospinal fluid, and vitreous humor) to investigate the presence of GHB in post-mortem samples and evaluate the possible benefit of testing additional specimens.

Materials and methods

Chemicals

Na-GHB and Na-GHB-d_6 (1 mg/mL in methanol) were obtained from Cerilliant (Round Rock, TX, USA). Methanol (Baker analyzed), ethyl acetate (Baker ultra resi-analyzed), and acetonitrile (Baker HPLC analyzed) were purchased from J.T. Baker (Devanter, Netherlands). N,O-bistrimethylsilyltrifluoroacetamide with 1 % trimethylchlorosilane (BSTFA+ 1 % TMCS) was purchased from Pierce (Rockford, IL, USA).

Calibrators and controls

Negative controls consisted of blank urine, serum samples, or 0.9 % saline (NaCl). Positive quality controls (QC) were prepared with concentrations of 20 mg/L (QC1) and 90 mg/L (QC2) in blank samples of urine, serum, or 0.9 % NaCl as controls for urine, blood samples, or cerebrospinal fluid/vitreous humor, respectively. GHB calibrators were prepared in demineralized water. The use of closely comparable matrices would be optimal. However, because of the presence of GHB in post-mortem blood samples, for this study, we did not use whole blood specimens for preparing control material.

Blank serum samples were provided by the department of transfusion medicine and blank urine samples were obtained from laboratory staff. All of the control specimens were analyzed for detectable GHB prior to further use.

Extraction procedure

Quantitative analysis was carried out via gas chromatography coupled to mass spectrometry (GC-MS) after extraction and derivatization with BSTFA+1 % TMCS [40]. Briefly, 50-μL aliquots of each specimen were spiked with GHB-d_6 as an internal standard (final concentration of 100 mg/L). A volume of 200 μL of HCl (0.1 mol/L) was added and samples were extracted with 1 mL of ethyl acetate for 5 min. After centrifugation (5 min; 12,000 rpm), the upper layer was transferred to conical bottom test tubes and evaporated for 30 min at 20 °C and 2 mbar in a vacuum centrifuge (Christ, Osterrode am Harz, Germany). The extract was dissolved with 50 μL of acetonitrile, and 25 μL of BSTFA+1 % TMCS was added. After mixing, the sample was immediately subjected to GC-MS analysis.

GC-MS analysis

GC-MS analyses were performed on a 5890A gas chromatograph coupled with an HP autosampler 6890 and a MSD 5970 with electron impact ionization (70 eV) (Hewlett Packard, Böblingen, Germany). A capillary column (30 m×0.25 mm, 0.25 μm) (VF-5 ms, factorFOUR, Varian, Darmstadt, Germany) was used with helium as a carrier gas (linear velocity 36.9 cm/s; carrier gas flow rate 1.0 mL/min; solvent delay 6.50 min). The injector temperature was 250 °C and the initial oven temperature was 80 °C for 2 min, and then the temperature was increased 13 °C/min up to 170 °C and 100 °C/min up to 300 °C. This temperature was maintained for 10 min (total run time 16.22 min). The MS was operated in selected ion-monitoring mode. Monitored ions (m/z) were as follows: GHB-di-TMS 233 (target), 204 (qualifier), and 234 (qualifier); and GHB-d_6-di-TMS (internal standard) 239 (target), 206 (qualifier), and 240 (qualifier). Retention times for the derivatives of GHB and the internal standard were 7.84 and 7.80 min, respectively.

Quantification of GHB

Quantification of GHB was performed by construction of five-point calibration curves (1.0, 10, 40, 80, and 100 mg/L), by calculating the ratio of the area of the molecular ion of GHB-di-TMS to the molecular ion of GHB-d_6-di-TMS as a function of the analyte concentration. Linearity of the resulting data was confirmed by regression analysis. One negative and two positive controls per specimen were analyzed within each run, and they showed acceptable quantitative results at both concentrations within ±20 % of the target value.

Validation parameters

The limit of detection was determined by estimating the minimum concentration that can be reliably distinguished from background noise (i.e., a signal that is three times the background noise while still allowing detection of ions in the correct ratio compared with control samples). The limit of quantification was defined as the lowest amount of an analyte in a control sample, which can be quantitatively determined with suitable precision and accuracy (i.e., coefficient of variation below 15 % and accuracy within 20 % of the expected result) [41, 42]. The limit of detection of the method was determined to be 0.5 mg/L and the limit of quantification was 0.6 mg/L, according to the abovementioned procedures. This method demonstrated good linearity (r^2>0.99) between 1.0 and 100 mg/L. There was no significant difference using a calibration curve prepared with water compared with a calibration curve in serum or saline. The recovery was determined with approximately 30 % for urine and serum and 19 % for cerebrospinal fluid and vitreous humor. The analytical procedure had an inter- and intra-day accuracy of 91 % or better and imprecision of 9 % or less, determined by analysis of two quality controls in duplicate for 8 days (according to the guidelines of the German Society of Toxicological Chemistry [42]). Selectivity of the method was assessed by examination of the endogenous compounds alpha-hydroxybutyric acid and beta-hydroxybutyric acid, 1,4-BD, and other drugs commonly occurring in post-mortem samples.

Samples

Heart blood, venous blood, urine, cerebrospinal fluid (liquor), and vitreous humor were collected during autopsy. In all autopsy cases, GHB exposure as the cause of death was excluded by a qualified forensic pathologist. Specimens were either analyzed on the day of sampling or were immediately stored at −20 °C until analysis. Additionally, in cases with

decomposition of the body, samples were collected and handled the same as other cases.

Stability of GHB at −20 °C

To investigate the long-term stability of GHB, in 10 cases, heart blood and peripheral blood were analyzed 24 months after the first measurement a second time.

Statistical analysis

GHB concentrations were compared using the Student's *t* test. Pairwise *t* tests were used for the long-term stability test. Pearson's correlation was used to detect associations between GHB and PMI, and between GHB concentrations in different specimens. All statistical calculations were conducted with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). *P* values less than 0.05 were regarded as significant. For concentrations below the limit of quantification, we used the value of zero.

Results

GHB concentrations in different specimens

During the sampling period (01/2009 to 11/2012), a sample size of 64 cases were collected. The PMI, which described the time between death and autopsy, ranged from 1 to 11 days (mean 4.8 days). The storage time between autopsy and GHB analysis ranged from 0 days to a maximum of 21 months at −20 °C.

Among the 64 cases, venous blood samples were available in 61, heart blood was available in 56, urine was available in 50, vitreous humor was available in 54, and cerebrospinal fluid was available in 52 cases. The range, mean, and median GHB values for each specimen are shown in Table 1.

The highest GHB concentrations (up to 65.3 mg/L) were measured in heart blood samples and the lowest GHB concentrations were measured in cerebrospinal fluid, followed by urine and vitreous humor (Fig. 1). In heart blood, 5 of 56 cases (9 %) showed GHB concentrations above 30 mg/L. In venous blood samples, GHB levels were slightly lower than those in heart blood, with no cases of endogenous GHB concentrations above 30 mg/L, and 87 % of cases (53/61) were below 20 mg/L (Table 1). In vitreous humor, most of the cases (85 %; 46/54) showed GHB concentrations below 20 mg/L, while three samples were 31.3, 31.5, and 39.0 mg/L GHB. In cerebrospinal fluid and urine, all GHB concentrations were below 30 mg/L. In cerebrospinal fluid, GHB levels were <10 mg/L in 92 % (48/52) of the cases. In urine, GHB concentrations were <10 mg/L in 76 % (38/50) cases, and in 94 % of cases (47/50), they were below 20 mg/L.

There was no significant difference in GHB concentrations among the five specimens. Correlations between different specimens were moderate for each comparison. The *r* value was 0.597 for the correlation between venous blood and heart blood, 0.530 between venous blood and vitreous humor, 0.498 between venous blood and urine, and 0.411 between venous blood and cerebrospinal fluid.

Post-mortem GHB concentration ratios were determined for each specimen compared with femoral vein blood (Table 2). The range of the ratios in each specimen was wide, but GHB concentrations in cerebrospinal fluid and urine were clearly below those found in peripheral blood samples (mean and median ratios <1.0). In contrast, GHB concentrations in vitreous humor and heart blood samples exceeded venous blood concentrations more than fourfold (e.g., cases 21 and 32).

In 10 autopsy cases with beginning or advanced putrefaction at the time of autopsy, urine (n=10), heart blood (n=9), venous blood (n=10), cerebrospinal fluid (n=7), and vitreous humor (n=9) were able to be collected. There was no difference in GHB concentrations in cases without putrefaction (cases 1–64) compared with those with putrefaction (cases 65–74). Because bodies at different stages of decomposition were examined, we divided cases into groups with beginning (greenish discoloration of the abdomen; group A) and advanced putrefaction (greenish and purplish discoloration of the skin, marbling pattern, decomposition gas; group B). GHB concentrations in each sample, the PMI, and stages of putrefaction are shown in Table 3.

Effect of pH values

The pH value was determined in case numbers 39 to 64. In 20 heart blood, 26 peripheral blood, and 25 urine samples, pH ranged from 5.9 to 6.4, 6.0 to 6.7, and 4.75 to 8.73, respectively. There was no or little correlation between GHB concentrations and pH levels for each specimen (r=−0.18 to −0.38).

Stability of GHB at −20 °C

There was no significant difference in the GHB concentrations of 10 heart blood samples and 10 venous blood samples, which were analyzed a second time 2 years after initial measurement (heart blood, P=0.779 and venous blood, P=0.312, respectively). The mean heart blood GHB concentration in samples was 21.6±16.1 mg/L (initial measurement) and 20.4 ±16.8 mg/L (second measurement). In peripheral blood samples, the first mean GHB concentration was 17.5±7.2 mg/L,

Table 1 GHB concentrations in different specimens and the PMI for all 64 cases

Case number	Heart blood (mg/L)	Venous blood (mg/L)	CSF (mg/L)	Vitreous humor (mg/L)	Urine (mg/L)	PMI (days)
1	*14.7*	12.4	n.a.	9.2	9.0	3
2	*13.8*	8.3	n.a.	7.1	2.8	3
3	*11.7*	7.5	1.0	1.0	2.1	2
4	6.3	*12.3*	<0.6	8.6	n.a.	8
5	*14.4*	12.9	<0.6	8.9	n.a.	6
6	*42.0*	23.6	1.3	13.9	20.3	6
7	*12.9*	10.9	4.0	11.4	n.a.	8
8	*7.8*	6.1	n.a.	1.8	2.3	2
9	*13.0*	9.4	2.9	11.8	<0.6	10
10	*14.5*	10.0	3.7	4.3	n.a.	3
11	*6.1*	5.2	4.6	2.2	<0.6	4
12	2.8	9.9	6.1	*14.9*	3.1	4
13	*14.1*	12.6	1.7	4.1	<0.6	2
14	*8.6*	5.8	<0.6	4.6	2.8	4
15	*8.6*	7.3	1.8	n.a.	<0.6	3
16	*22.8*	11.4	2.6	20.1	1.1	9
17	*25.6*	12.1	<0.6	8.9	13.4	4
18	<0.6	<0.6	n.a.	<0.6	<0.6	5
19	2.7	*12.9*	<0.6	4.3	<0.6	3
20	17.1	13.3	9.2	*20.6*	n.a.	2
21	*25.0*	5.9	n.a.	14.9	n.a.	2
22	10.0	*24.9*	n.a.	8.5	15.2	5
23	24.9	28.4	n.a.	*39.0*	<0.6	5
24	*65.3*	26.5	10.4	n.a.	21.4	7
25	15.5	*17.5*	n.a.	<0.6	2.0	2
26	*26.0*	10.6	9.1	8.4	n.a.	5
27	13.8	*21.8*	<0.6	3.1	<0.6	2
28	*15.0*	12.4	3.8	n.a.	<0.6	5
29	10.4	*17.3*	<0.6	<0.6	n.a.	1
30	*31.6*	16.8	3.2	31.5	n.a.	11
31	8.8	11.2	9.1	*13.5*	n.a.	9
32	15.2	6.6	4.4	*27.8*	n.a.	7
33	9.8	*10.0*	1.8	9.0	n.a.	7
34	12.1	*13.1*	n.a.	4.3	n.a.	2
35	*9.4*	n.a.	<0.6	n.a.	5.6	3
36	*30.1*	n.a.	10.8	25.5	25.1	6
37	n.a.	28.7	24.0	*31.3*	10.6	2
38	n.a.	n.a.	<0.6	*17.5*	n.a.	7
39	7.2	*10.5*	1.0	4.3	3.6	2
40	*9.2*	7.6	n.a.	2.8	4.4	2
41	*12.3*	5.7	3.3	3.0	9.8	11
42	12.4	*12.9*	1.4	n.a.	8.8	8
43	*22.6*	14.7	9.9	n.a.	10.0	7
44	*19.4*	14.5	n.a.	n.a.	18.0	7
45	*6.5*	3.9	2.3	2.1	1.2	3
46	*15.3*	4.9	5.8	n.a.	4.4	4
47	*10.3*	10	4.1	n.a.	9.1	3
48	*12.6*	10.5	4.8	11.2	11.1	8
49	*15.7*	14.7	6.5	10.5	6.9	6
50	8.9	10.2	*10.3*	n.a.	5.7	2
51	n.a.	*8.1*	3.8	4.1	n.d.	3
52	n.a.	*7.9*	2.8	3.1	5.6	2
53	n.a.	8.8	4.7	6.4	*14.6*	6
54	11.7	*15.8*	5.1	6.9	4.2	5
55	*33.2*	22.0	n.a.	21.9	15.7	10
56	n.a.	*6.8*	1.6	2.5	1.3	2
57	n.a.	10.7	2.9	5.0	*10.8*	4
58	*15.7*	8.2	3.9	6.6	n.d.	5
59	*8.3*	7.2	2.5	3.1	n.d.	2
60	n.a.	*11.1*	n.a.	7.8	3.6	6
61	16.5	*20.1*	n.a.	8.7	6.9	5
62	7.5	7.7	4.6	*8.6*	4.0	7
63	*10.7*	7.9	3.1	5.3	3.3	5
64	*11.4*	4.8	2.7	1.7	n.d.	1
n	56	61	52	54	50	
Range	<0.6–65.3	<0.6–28.7	<0.6–24.0	<0.6–39.0	n.d.–25.1	1–11
Mean	15.2	11.9	4.2	9.6	6.0	4.8
Median	12.8	10.6	3.2	7.5	3.8	4.5

Specimens with the highest concentrations are in italics for each case. Ranges, means, and medians were calculated

n.a. not available, *n.d.* not detected (GHB concentration was below the limit of detection), *PMI* post-mortem interval (time between death and sample collection), *<0.6* GHB concentration was below the limit of quantification

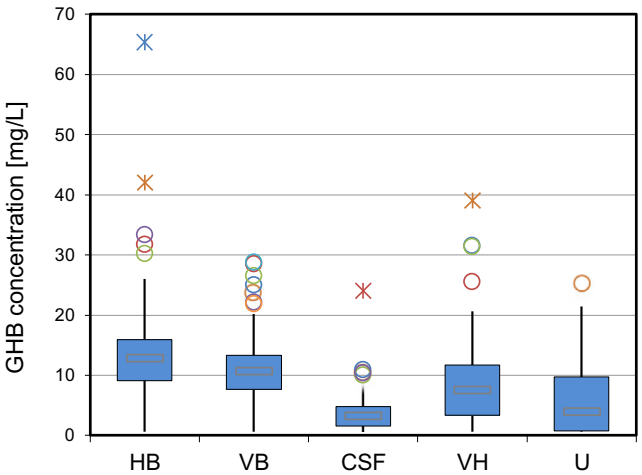

Fig. 1 GHB concentrations (mg/L) in different body fluids. Specimens are displayed as *box plots*. Data outside the 1.5 interquartile range are displayed as *circles* and are *outliers*; data outside the threefold interquartile range (*stars*) can be marked as extremum. *HB* heart blood, *VB* venous blood, *CSF* cerebrospinal fluid, *VH* vitreous humor, *U* urine

Table 2 Post-mortem GHB concentration ratios relative to femoral vein blood (VB)

	HB/VB	CSF/VB	VH/VB	U/VB
n	54	47	52	48
Range	0.22–4.21	0.03–1.17	0.03–4.21	0.02–1.72
Median	1.19	0.37	0.70	0.37
Mean	1.35	0.40	0.81	0.50

The range, median, and mean ratios for heart blood (HB), cerebrospinal fluid (CSF), vitreous humor (VH), and urine (U) are shown

and after 24 months, the mean GHB concentration of 10 samples was 15.2±6.4 mg/L (Fig. 2a, b).

Discussion

GHB concentrations in different specimens

In our study, no homogenous pattern of post-mortem GHB concentrations was determined, but there appeared to be a trend. GHB concentrations were highest in heart blood in more than half (56 %; *n*=36) of all cases. In 25 % (16/64) of the cases, venous blood samples showed the highest GHB concentrations. Urine GHB concentrations were low compared with those in blood. The highest GHB concentrations in urine were in the same range of corresponding blood samples (Table 1).

In most of our cases, GHB concentrations in the vitreous humor were below or in the same range as those in blood specimens. In 11 % (*n*=7) of our cases, GHB concentrations in the vitreous humor exceeded GHB venous and heart blood concentrations. However, in 1 of our cases, no blood samples were available (case 38). GHB concentrations in cerebrospinal fluid were generally lower than in other specimens, but in case 50, GHB concentrations in cerebrospinal fluid and venous blood were equal.

Comparison of post-mortem GHB concentrations

Results of other studies on post-mortem GHB concentrations were compared with our findings. A summary of published GHB concentrations that were measured in post-mortem biological fluids in fatalities without GHB involvement is shown in Table 4.

In urine, post-mortem GHB concentrations that were determined in other studies were lower than those in blood specimens, similar to our samples [37, 38]. This finding was particularly prevalent in studies with high GHB blood concentrations [27, 35].

The range of endogenous GHB concentrations in the vitreous humor as determined by Kintz et al. and Marinetti et al. were 3.9–21.4 and 0–7 mg/L, respectively, and agreed with our results (Table 4) [28, 35]. Additionally, Kugelberg et al. determined GHB concentrations in three cases of GHB intoxication. In the vitreous humor, they detected GHB concentrations in the same range as in femoral blood samples [14].

With regard to cerebrospinal fluid, there are little published data on GHB concentrations with which to compare with our study. Moriya and Hashimoto showed that GHB concentrations in cerebrospinal fluid and vitreous humor were similar to those in urine samples, and significantly lower than those found in femoral venous blood (Table 4) [37]. Mean GHB concentrations in their study were 1.8 mg/L in cerebrospinal

Table 3 GHB concentrations in different specimens and PMIs in 10 autopsy cases with signs of putrefaction

Case number	Heart blood (mg/L)	Venous blood (mg/L)	CSF (mg/L)	Vitreous humor (mg/L)	Urine (mg/L)	PMI (days)	Degree of putrefaction
65	20.1	21.5	6.7	16.8	*26.7*	5	A
66	20.2	18.2	5.3	*31.0*	0.8	2	A
67	*14.6*	10.6	2.4	11.0	5.3	13	A
68	5.8	2.3	*6.4*	4.3	5.8	4	A
69	*11.4*	8.2	5.7	5.3	6.9	3	A
70	*28.4*	17.6	6.0	13.4	6.3	8	A
71	23.1	*32.7*	n.a.	n.a.	18.8	9	B
72	*8.2*	7.1	2.6	3.2	7.0	2	A
73	*5.5*	5.1	n.a.	3.5	1.7	4	A
74	n.a.	*14.9*	n.a.	13.0	7.2	5	A
n	9	10	7	9	10		
Range	5.5–28.1	2.3–32.7	2.4–6.7	3.2–31.0	0.8–26.7	2–13	
Mean	15.2	13.8	5.0	11.3	8.6	5.5	
Median	14.6	12.7	5.7	11.0	6.6	4.5	

The specimen with the highest concentration measured is in italics for each case. GHB concentration ranges, means, and medians were calculated

n.a. specimen not available, *A* beginning putrefaction (greenish discoloration of the abdomen), *B* advanced putrefaction (greenish and purplish discoloration of the skin, marbling pattern, and decomposition gas)

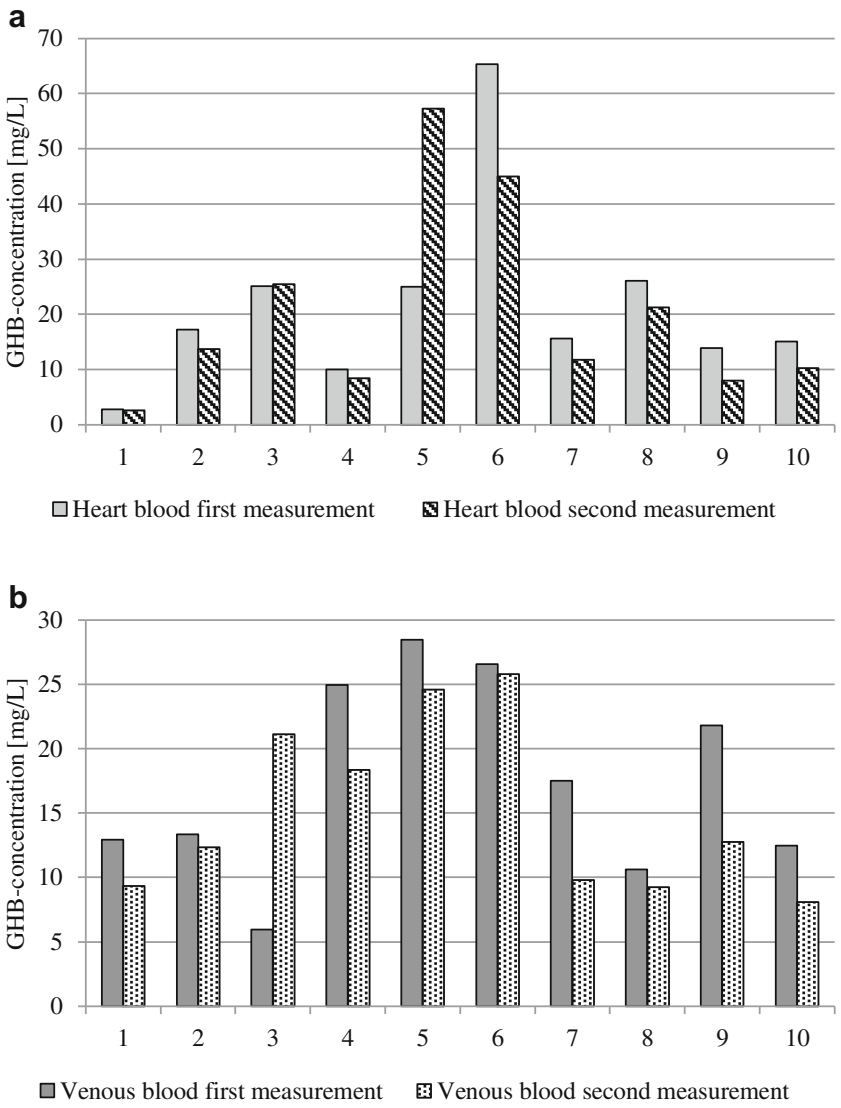

Fig. 2 GHB concentrations (mg/L) in 10 heart blood samples (**a**) and peripheral blood samples (**b**). Samples were measured a second time 24 months later

fluid (n=9) and 0.9 mg/L in vitreous humor (n=8) [37]. These values are lower than those determined in our study (means 4.2 and 9.6 mg/L). Because GHB concentrations in this study [37] in post-mortem blood (0–11.6; mean 4.6 mg/L; n=23) were lower than other results (Table 4), an ethnic influence should be considered, as suggested in our study on ante-mortem specimens [26]. In post-mortem venous blood samples, GHB concentrations in our study were lower than those in previous studies. Fieler and co-workers reported GHB concentrations up to 168 mg/L in post-mortem venous blood samples [27] and another study showed up to 197 mg/L [29]. GHB concentrations in peripheral blood samples in our study were lower, with a maximum concentration of 28.7 mg/L (case 37).

For heart blood samples, GHB concentrations as high as 409 mg/L were observed in samples from persons without exogenous GHB uptake [28], and other studies have shown up to 119 mg/L [35] and 116 mg/L [43]. In our study, even heart blood GHB concentrations were lower than those in previous studies, with a maximum GHB concentration of 65.3 mg/L (case 24).

These considerable differences in GHB concentrations can be partly explained by different methods of analysis. Elliott determined GHB concentrations by a GC-MS method with diethylene glycol as the internal standard. He concluded that the high GHB post-mortem levels compared with data of other authors could be attributed to a non-optimal analytical procedure [29, 38]. He also assumed that the exceptionally high concentrations determined by Fieler et al. [27] could be explained by the methodology of the analysis.

Despite these methodological differences, the main cause of high post-mortem GHB concentrations is the storage condition of samples before analysis. In a study by Kintz et al., specimens (venous blood, urine, and vitreous humor) were delivered within 48 h of autopsy to the laboratory and were stored at 4 °C until analysis [28]. They found that the

Table 4 Summary of published GHB concentrations in post-mortem biological fluids in fatalities without GHB involvement

Authors	PMI[a]	Storage conditions	Analytical method	GHB concentrations (mg/L)			
				Peripheral blood	Heart blood	Urine	Other specimens
Fieler et al. 1998 [27]	n.i.	n.i.	GC-FID and GC-MS via conversion to GBL I.S. DVL	0–*168*[d] (*n*=20)		<1 (*n*=8)	
Stephens et al. 1999 [39]	n.i.	4 °C up to 60 days or 25 °C, with a mean of 40 days	GC-MS via conversion to GBL I.S. DVL	4 °C, <5–*77* and 25 °C, 9–*433* (*n*=20)			
Couper and Logan 2000 [5]	n.i.	n.i.	GC-MS I.S. DEG	3–*107*[d] (*n*=17)			
Kalasinski et al. 2001 [11]	n.i.	n.i.	GC-MS I.S. $GHBd_6$	3–14[c] (*n*=12)			
Elliott 2001 [29]	<4 days	4 °C for 4 days, −20 °C for <6 months	GC-MS and GC-FID I.S. DEG	0–*197*[d] (*n*=13)		0–217 (*n*=13)	VH: positive (*n*=13)
Sakurada et al. 2002 [33]	5–7 days	7 days–1 year at −20 °C	GC-MS I.S. DEG	<0.4–7.3[d] (*n*=5)		0–2.6 (*n*=5)	Liver: 2.6–12 (*n*=5)
Elliott 2004 [4]	2–62 days[b]	4 °C, analysis within 10 days	GC-MS I.S. $GHBd_6$	0–29 (*n*=38) and 0–25 (*n*=17)[c]		0–18 (*n*=39)	VH: 1–3 (*n*=2)
Moriya and Hashimoto 2004 [30]	8–132 h	4 °C for 1 day–15 months until long-term storage at −20 °C for 10 days–7 months	HS-GC-FID via conversion to GLB	0–*43* (*n*=43)			
Kintz et al. 2004 [28]	12–72 h	4 °C, samples arrived within 48 h of autopsy	GC-MS I.S. $GHBd_6$	17–*44* (*n*=5)	0.4–409 (*n*=71)		VH: 3.9–21.4 (*n*=6)
Marinetti et al. 2005 [35]	Maximum of 24 h	4 °C for 1.5–4 months, followed by long-term storage at −15 °C for 19–50 months	GC-MS I.S. $GHBd_6$	0–*97* (*n*=26)	0–119 (*n*=26)	0–7 (*n*=26)	VH: 0–7 (*n*=26)
Moriya and Hashimoto 2005 [37]	6–48 h	−20 °C or analysis shortly after autopsy	HS-GC-FID via conversion to GLB	0–11.6 (*n*=23)	0–15.8 (*n*=21)	0.6±1.2 (*n*=12)	VH: 0.9±1.7 (*n*=8) and CSF: 1.8±1.5 (*n*=9)
Erdmann et al. 2006 [43]	0–14 days	n.i.	GC-MS I.S. $GHBd_6$		2.2–116 (*n*=50)		

Concentrations above 30 mg/L in peripheral blood samples are in italics

DEG diethylene glycol, *DVL* delta-valerolactone, *GC-FID* gas chromatography/flame ionization detection, *HS-GC-FID* head-space gas chromatography with flame ionization detection, *n.i.* no information available

[a] Time between death and autopsy/sample collection

[b] Time between death and analysis

[c] Sample with preservative (NaF)

[d] No information if heart blood or peripheral blood samples were investigated

maximum GHB concentration was 44 mg/L in peripheral blood. Marinetti et al. first stored samples (venous blood, heart blood, urine, and vitreous humor) at 4 °C for 1.5–4 months before long-term storage in a freezer and determined a maximum GHB concentration of 97 mg/L in venous blood [35]. In another study regarding GHB stability, a maximum GHB concentration of 433 mg/L was shown in venous blood samples. The specimens were stored at room temperature for up to 60 days (mean 40 days) [39]. Moriya and Hashimoto stored venous blood samples at 4 °C for 1 day to 15 months until long-term storage at −20 °C, with a maximum GHB concentration of 43 mg/L [30].

Marinetti et al. [35] and Elliott [38] reported the results of a study by Anderson and Kuwahara [44], who found GHB concentrations in femoral blood samples ranging from 1.7 to 48 mg/L (mean 11 mg/L). Unfortunately, there is no information regarding the analytical method and storage conditions [44]. Results of Elliott [38], Moriya and Hashimoto [37], and Sakurada et al. [33] were within the range of our GHB concentrations in peripheral blood specimens. Other studies that were summarized by Elliott [38] were also in line with GHB concentrations in our study, including 0–21 mg/L in 37 peripheral blood samples [45], 5–20 mg/L in a number of specimens out of a sample of 21 cases [46], and 3–14 mg/L in 12 control cases [47].

Value of testing other specimens in addition to venous blood

Some authors recommend analysis of urine in addition to blood samples because of typically (but not generally) [38] lower GHB concentrations due to less post-mortem generation [27]. Other authors have suggested the vitreous humor as the specimen of choice, in addition to femoral vein blood [28, 35, 37].

In our study, GHB concentrations in urine and cerebrospinal fluid were lower than those in peripheral blood samples and measurement of these specimens would be suitable for accurate determination of endogenous GHB concentrations (Fig. 1). Therefore, in contrast to Kintz et al. [28], we recommend that urine should be analyzed in addition to peripheral blood. Our results support the findings of previous studies that heart blood samples are not sufficient for quantitative analysis in post-mortem toxicology and determination of GHB concentrations solely in heart blood may lead to misinterpretation.

Measurement of GHB in the vitreous humor is useful in most cases, but GHB concentrations in the vitreous humor could exceed concentrations in blood (Table 1; cases 12, 20, 23, and 32). In some cases, GHB concentrations in the vitreous humor were the highest measured but were comparable with venous blood (cases 31, 37, and 62). Therefore, if possible, the vitreous humor should not be the only specimen measured in addition to venous blood.

In some post-mortem cases, it is not possible to obtain urine samples, as found in our study (venous blood samples, $n=61$; urine samples, $n=50$). Taking cerebrospinal fluid and vitreous humor for toxicological analysis is not always possible and requires experienced staff. Nevertheless, a reliable interpretation of GHB post-mortem results would be improved by additional analysis of cerebrospinal fluid, vitreous humor, and/or urine.

Stability of GHB at −20 °C

Recently, Fjeld et al. investigated the long-term stability in 16 GHB-positive post-mortem whole blood samples, stored for 0.5 to 7.2 years at −20 °C with sodium fluoride preservation. They found a change in GHB concentrations of +34.4 to −30.4 %, with a mean change of −7.1 %. They concluded that their study did not show changes in GHB concentrations of practical significance [48]. Another study showed that long-term frozen storage does not result in considerable formation of endogenous GHB [35]. This supports the contention that long-term storage at −20 °C should not considerably affect GHB concentrations in a given sample from the time it was placed in storage [35]. In frozen blood samples without any preservatives, there is no spontaneous formation of GHB during storage for at least 12 months. Therefore, GHB concentrations measured in immediately frozen blood samples reflect those present at autopsy [30].

In our study, no significant change in GHB concentrations was observed after storage at −20 °C for 24 months. In 1 case, GHB concentrations in heart blood were more than doubled at re-analysis. In 9 other cases, GHB concentrations slightly decreased but remained at the same range at re-analysis (2.8–65.4 vs. 2.6–57.3 mg/L; $n=10$). In venous blood, GHB concentrations increased in 1 case, and in 9 other cases, they decreased, but were at a comparable level (12.9–28.5 vs. 9.4–25.8 mg/L; $n=10$). All GHB concentrations in peripheral blood samples were below 30 mg/L before and after long-term storage. Only 10 cases were re-analyzed in our study. Further studies need to be conducted to generate data for the stability of GHB at −20 °C in urine, vitreous humor, and cerebrospinal fluid.

Effect of PMI

In our study, no relationship between PMI and endogenous GHB concentrations for venous blood, heart blood, and cerebrospinal fluid was found. There was a slight correlation between GHB concentrations and PMI in urine and vitreous humor specimens ($r=0.476$ and 0.407). Moriya and Hashimoto calculated a moderate positive correlation between GHB blood concentrations and PMI at a maximum of 48 h ($r=0.571$) [30]. They found no correlation between concentration and storage interval at 4 or −20 °C. Therefore, they concluded

that a large portion of endogenous GHB detected in blood of corpses may be produced during the interval between death and autopsy, rather than during storage at 4 °C until analysis.

Our data support their assumption that substantial amounts of GHB may be produced in blood as early as several hours after death, because at the time of sampling (one or more days after death), nearly all peripheral blood GHB concentrations (59/61) were above ante-mortem interpretative cut-offs (4 mg/L [26]). Most changes in the body occur during the first few hours after death and they will have been completed at the time of autopsy. An effect of a PMI beyond 1 or 2 days is not expected.

Effect of storage conditions

GHB concentrations in this study were considerably lower than those in most other previous studies. As discussed above, we speculate that a relevant increase in GHB will occur during storage at 4 °C or at room temperature. This possibility is supported by findings in a study by Moriya and Hashimoto who detected GHB concentrations up to 43 mg/L in peripheral blood samples (n=43), which were stored for at least 1 day at 4 °C before analysis [30]. In a later study by the same authors, storage conditions were optimized to immediate analysis or storage at −20 °C, and lower concentrations of GHB were measured with 0–11.6 mg/L in 23 post-mortem samples [37].

Effect of putrefaction

In cases with signs of putrefaction at the time of autopsy, GHB concentrations in different specimens were not significantly higher than those in cases without obvious signs of putrefaction (Table 3).

In all specimens, GHB concentrations were below 30 mg/L, even in heart blood. In only one case (no. 71), the GHB concentration in a venous blood sample was 32.7 mg/L. After a post-mortem period of 9 days, this case showed advanced decomposition. Notably, in seven of these cases, blood specimens (heart or venous blood) showed the highest concentration, but in one case within each group of specimens, GHB concentrations in cerebrospinal fluid, vitreous humor, and urine exceeded blood concentrations. Elliott et al. [49] found that GHB concentrations were 3–27 mg/L in five putrefied post-mortem blood samples, which is similar to our findings. In their study, they also could not detect a proportional relationship between GHB concentrations and the extent of putrefaction [49].

Cut-off recommendations

Because of the problem that GHB is an endogenous substance in all body fluids in which GHB concentrations will increase after death, a classic cut-off level (e.g., based on receiver operating characteristics curves), cannot be calculated. Moreover, the sensitivity and specificity of the suggested thresholds could not be calculated in our study, because in this cohort, all of the GHB concentrations were classified as *false positive* as we wanted to differentiate between exogenous and endogenous GHB. Nevertheless, we used our data to examine the recommendations of previous studies to facilitate application of an interpretative cut-off.

The suggested cut-off of 30 mg/L for post-mortem blood samples [37, 38] is supported by our results. GHB concentrations did not exceed 30 mg/L in 61 venous blood samples. However, notably, this cut-off cannot be applied for heart blood samples, because in 9 % (5/56) of the samples, GHB concentrations were >30 mg/L. Increasing the cut-off up to 50 mg/L for peripheral blood samples as suggested by Kintz et al. [28] is not necessary, provided that the samples are stored at −20 °C until analysis. Even for heart blood samples, a cut-off of 50 mg/L would not enable discrimination between endogenous and exogenous GHB. A cut-off of 30 mg/L can also be adopted for urine and cerebrospinal fluid samples. GHB concentrations in vitreous humor could exceed this level in rare cases (5.5 %). Therefore, for this specimen, the proposed cut-off of 50 mg/L [28] should be used. The recommended cut-off levels for post-mortem urine samples of 10 mg/L [37, 50] and 20 mg/L [38] are too low because 24 % of our cases (n=50) showed GHB concentrations above 10 mg/L, and at least 6 % exceeded the level of 20 mg/L. We propose to use 30 mg/L as the interpretative cut-off for urine.

Previously published reports of higher GHB concentrations exceeding the level of 30 mg/L in peripheral blood samples of cases without the contribution of exogenous GHB appear to be mainly owing to (inappropriate) sample storage in non-frozen conditions. Therefore, we intend to perform an additional study to investigate the course of in vitro formation of GHB during non-appropriate storage conditions and the possible influence of preservatives will be tested. Additionally, the effect of GHB-glucuronide on endogenous post-mortem GHB concentrations should be investigated. Of course, despite any cut-off recommendations, each post-mortem case has to be interpreted holistically, with respect to all aspects of the case.

Conclusion

In conclusion, an interpretative cut-off of 30 mg/L for GHB concentrations can be adopted for the post-mortem specimens of peripheral blood, urine, and cerebrospinal fluid. In several heart blood samples, and even in the vitreous humor, higher GHB concentrations are present. This recommendation is only applicable in cases where GHB analysis is conducted on the day of sample collection or after immediate storage at −20 °C. Extended PMIs (>24 h) did not significantly affect GHB

levels in our study cohort. However, in corpses with advanced putrefaction, an increase in GHB in venous blood samples is possible. GHB concentrations above 30 mg/L in peripheral blood samples that were published by other authors appear to mainly be the result of generation during storage. Therefore, appropriate sample handling is of utmost importance in GHB analysis.

Conflict of interest The authors declare that they have no conflict of interest.

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