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Several possible toxicological and genetic tools for the extension of the detection window after GHB intake

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Aims: Because of its short detection window, uncovering the intake of gamma-hydroxybutyric acid (GHB) still constitutes a problem. Aim of the experiments was to develop and evaluate new tools for a possible extension of the detection window after the intake of GHB. **Methods:** Blood, plasma and urine samples (each n=49) of volunteers and of patients (n=3, patient 1 and 2 chronical intake, patient 3 single intake) therapeutically taking up to 4.5 g GHB (Xyrem®) per night were collected at different points in time after the intake up to 72 h. Additionally, hair samples of the patients were taken. Concentration profiles of GHB (high-performance liquid chromatography-tandem mass spectrometry), GHB-β-glucuronide and GHB-4-sulfate (high-performance liquid chromatography-quadrupole time-of-flight-mass spectrometry) in plasma and urine were recorded over time. Hair samples were analyzed by a validated LC-MS/MS method for GHB and GHB-β-glucuronide. Alterations in gene expression of *ALDH5A1*, *AKR7A2*, *EREG* and *PEA15* in blood, genes of interest which code for enzymes involved in GHB metabolism, was investigated via quantitative PCR using an empirically derived normalization strategy. Furthermore, possible discrimination of endogenous from exogenous GHB in urine via isotope ratio MS was tested. **Results and discussion:** The parent compound could be quantified above the usual cut-offs for 4-6 hours both in blood and urine. No discrimination endogenous/exogenous by neither using the phase II metabolites of GHB nor using the expression of the genes of interest was possible. In the hair samples of patients GHB and its glucuronide could not be determined in concentrations higher than the control group. A discrimination endogenous/exogenous GHB was only possible using isotope ratio mass spectrometry, however carbon isotope ratios in urine did not differ longer than GHB was detectable above the cut-off limit. **Conclusion:** Therefore, these methods do not seem to be able to extend the detection window of exogenous GHB.

1. Introduction

Gamma-hydroxybutyric acid (GHB) is a central nervous system depressant which is generated endogenously originating from the neurotransmitter GABA (gamma-aminobutyric acid) via the enzymes GABA-transaminase and succinat-semialdehyde-reductase [1]. Apart from its natural occurrence, GHB is therapeutically taken for the treatment of narcolepsy [2] and its use emerged as a so-called knockout-drug for drug facilitated crimes (DFCs) [3].

Uncovering DFCs related to GHB has been difficult, due to its very narrow detection window in plasma (<6 h) and urine (<12 h) depending on the ingested dose [4, 5]. Commonly used cutoff values in ante mortem samples are 4 mg/L for plasma and 6 mg/L for urine [6]. In 2013, the GHB metabolite GHB-β-*O*-glucuronide (GHB-Gluc) in urine samples was described by Petersen et al. [7] and Hanisch et al. [8] described the sulfonated GHB (GHB-Sulf) in urine samples in 2015.

To date only few studies applying alternative methodologies to verify GHB exposure and to widen the window of detection are available. Saudan et al. [9] employed gas chromatography isotope ratio mass spectrometry (GC/C/IRMS) to determine the differences in carbon isotope composition between exogenous and endogenous GHB in blood samples of human subjects. Furthermore, hair analyses of human subjects after GHB intake were investigated by several research groups [10-12]. A new approach was conducted by Larson et al. [13] with the aim to identify a surrogate marker for GHB intake. They showed that the genes *PEA-15* and *EREG* were significantly higher expressed in mice which received an intraperitoneal GHB injection. The aim of the present study was to find a new potential approach for the determination of an exogenous GHB intake via toxicological, biochemical or genetic investigations.

2. Material and Methods

2.1. Study design

The study design was approved by the regional ethics committee of the University Hospital of Bonn according to the declaration of Helsinki (number: 370/13). The volunteers were informed about the risks of the study and gave their written informed consent. Biological specimens were collected from volunteers with (*case group, n*=3) and without (*control group, n*=49) GHB-intake.

2.1.1. Case group

Participants were patients suffering from narcolepsy (*n*=3). The first two volunteers took daily the pharmaceutical Xyrem® for more than five years. Test person 1 and 2 stopped taking the medication 3 days and 18 h before the experiment started, respectively. For the excretion study P1 and P2 took the pharmaceutical Xyrem® with a dose of 2.25 g (1.87 g GHB). Subsequently whole blood and urine samples were collected prior to and after 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 48 and 72 h and were stored at -20°C. Test person 3, diagnosed as narcolepsy, was drug free before participating in the trial. Whole blood in PAXgene tubes, plasma and urine samples of P3 were collected after a single dose administration of 2.42 g sodium oxybate solution (volume: 10 mL, 2 g GHB, Somsanit®) according to the protocol described above. Sample collection of P3 finished 24 h after GHB exposure.

Hair samples were collected from the back of the head from each patient and stored at room temperature. The hair sample of P1 was 37 cm long and bleached; original hair colour was dark blond. P2 had 50 cm long, brown hair; the original hair colour was light brown. The distal 20-25 cm of the hair strand were dyed with brown hair colour and were not involved in the investigation. The hair of P3 was black and without hair treatment (hair length: 6 cm).

2.1.2. Control group

Blood in PAXgene tubes, plasma and urine samples were collected from volunteers (*n*=49) who had never taken GHB during their lives and stored at (-20°C and -80°C for RNA) until

analysis. Control group did not differ significantly from the three cases in age (p>0.05) and BMI (p>0.05) by assessing the Mann-Whitney-U-test.

2.2. Materials and Methods

GHB and its metabolites GHB-Gluc and GHB-Sulf were analysed and determined in plasma and urine samples via high-performance liquid chromatography-tandem mass spectrometry (LC/MS/MS) and high-performance liquid chromatography-quadrupole time-of-flight-mass spectrometry (LC-QTOF-MS). Method details have been published elsewhere [14, 15]. The approach to measure exogenous GHB in urine using isotope ratio mass spectrometry (IRMS) was also previously published [16]. Alterations in gene expression of the genes *ALDH5A1*, *AKR7A2*, *EREG* and *PEA15* after GHB intake were analyzed by quantitative Realtime-PCR. *ALDH5A1* and *AKR7A2* were chosen, because they code for enzymes involved in GHB metabolism. Former experiments showed that the expression of the genes *EREG* and *PEA15* increased in mice when GHB was injected [13]. Therefore, *EREG* and *PEA15* were also investigated. An empirically derived normalization strategy was established for this approach. Method details were described in Mehling et al. Int J Legal Med "Alterations in gene expression after gamma-hydroxybutyric acid intake - a pilot study*" (accepted may 2017)*.

3. Results and Discussion

3.1. Toxicological Investigations
3.1.1. Analysis in plasma and urine

We investigated GHB and GHB-Gluc concentrations in plasma and urine samples of 49 controls and compared the results with plasma and urine samples of three patients taking GHB for the indication of narcolepsy [17].

For the three positive cases excretion patterns are shown in Figure 1. GHB plasma concentrations of all patients decreased and fell below the commonly applied cutoff value 4 h after ingestion (Fig. 1, left). In urine, GHB concentrations were measured below the cutoff value of 10 mg/L after 6 h in P1 and P3 as well as after 8 h in P2 (Fig. 1, right). Results were previously published [17].

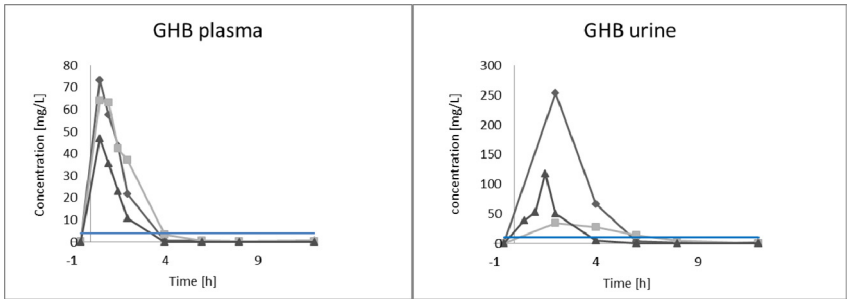


Fig. 1. Gamma-hydroxybutyric acid (GHB) concentrations of the three test persons after ingestion of sodium oxybate plotted against collected time in plasma (left) and urine (right). (▬) marks the commonly used cutoffs in plasma (<4 mg/L). *P1* first (–♦–), *P2* second (–■–), *P3* third test person (–▲–).

Results of GHB-Gluc in urine and plasma were already published elsewhere [17]. Briefly, in Figure 2 GHB-Gluc concentration-time profiles for plasma of the three test-persons (P1-3) are displayed. The underlying lines depict the range of determined endogenous plasma GHB-Gluc concentrations in the control group. In general, GHB-Gluc concentrations showed no time-depended increase after GHB intake (Fig. 2). GHB-Gluc concentrations of the P2 and P3 were in the same range as the control group. P1 showed higher GHB-Gluc concentrations in plasma, which were determined above the maximum of the 49 volunteers of the control

group. The GHB-Gluc concentration of P1 even before GHB administration was above the maximum level of the control group.

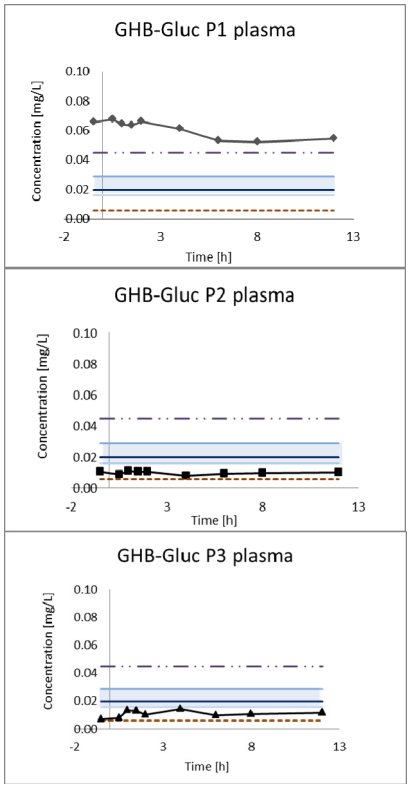
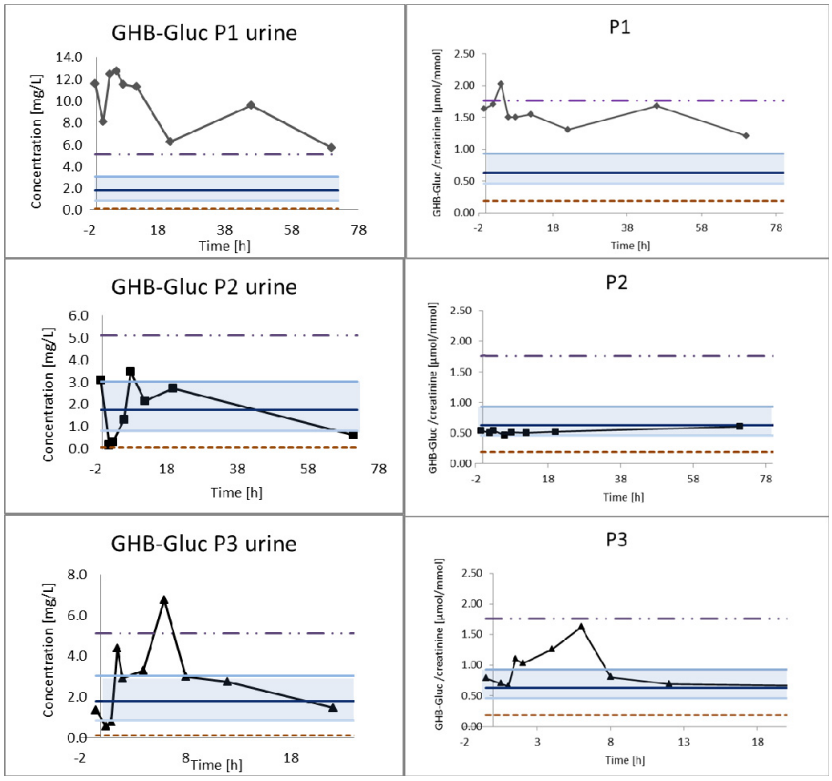


Figure 3 (below) shows the GHB-Gluc concentrations of the case group (P1-3) compared to the ranges of endogenous GHB-Gluc concentrations without GHB-intake in urine samples. In addition, urinary GHB-Gluc concentrations normalized by creatinine concentrations are also depicted (right side). For P1, GHB-Gluc concentrations were determined above the maximum concentration of the control group (5.09 mg/L). The measured concentrations of P2 and P3 were almost within the endogenous concentration range in urine samples. Before GHB ingestion GHB-Gluc concentrations were determined to be 11.56, 3.12 and 1.34 mg/L for P1, P2 and P3, respectively. After GHB intake GHB-Gluc concentrations increased in all test persons, but the determined elevated levels were almost within the studied range of the control group without GHB exposure.

Fig. 2. GHB-Gluc concentrations in plasma samples of the three test persons compared to endogenous plasma concentrations of the control group. The blue area shows the endogenous concentrations between the first and third quartile (Q1= first quartile (—); Q3= third quartile (—)) of the control group. Below and above the blue area, lines for minimum (– –) and maximum (– · ·) of the endogenous plasma concentrations are drawn together with the median (—); *P1* first (–♦–), *P2* second (–■–), *P3* third test person (–▲–).



Maximum GHB-Gluc concentrations were determined after 6 h (P1, P3) and 8 h (P2).

Fig. 3. GHB-Gluc concentrations of the three test persons in urine compared to endogenous urine concentrations of the control group with (right) and without (left) normalization to creatinine. The blue area shows the endogenous concentrations between the first and third quartile (Q1= first quartile (—); Q3= third quartile (—)) of the control group. Below and above the blue area lines for minimum (– –) and maximum (– · ·) of the endogenous plasma concentrations are drawn together with the median (—); *P1* first test person (–♦–), *P2* second test person (–■–), *P3* third test person (–▲–).

After normalization with creatinine concentrations the values for GHB-Gluc of P1 were above the third quartile, but largely below the maximum endogenous concentration of the control

group. Furthermore, an increase of GHB-Gluc values could also be observed in P1 and P3 after normalization by creatinine concentrations in urine, contrary to P2, where no increase was observed. Due to drinking of relatively large volumes of water after GHB administration the urine samples of P2 at the time-points 2 and 4 h were highly diluted. Therefore, less GHB, GHB-Gluc and creatinine concentrations were found in these urine samples.

The determined GHB-Gluc concentrations of the three test persons in plasma and urine showed no noteworthy results compared to the control group. Almost no time-depended increases of the GHB-Gluc concentrations exceeding the maximum level of the control group were determined in plasma and urine of P2 and P3 after GHB intake. Hitherto, the GHB-metabolite GHB-Gluc seems not to be a suitable marker in plasma and urine to extend the detection window after GHB intake [17].

Results about the suitability of GHB-sulfate as a potential marker to uncover a GHB intake will soon be published. The article is still within publication process (Piper et al. submitted to Forensic Sci. Int. "*Potential of GHB-4-sulfate to complement current approaches in GHB post administration detection").*

3.1.2. Hair analysis

The investigations on hair showed that a single GHB exposure might not be determined by hair analysis of GHB and GHB-Gluc (P3). The chronical intake of therapeutic sodium oxybate was also not confirmed by hair analysis maybe due to hair treatments (P1 and P2). Regardless of sampling time or pre-analysis treatment of the hair no increased concentrations of GHB and its glucuronide were detected. Therefore, GHB hair analysis should be assessed critically and determined negative results cannot exclude GHB exposures, because chronical therapeutic use could lead to endogenous GHB hair concentrations. Further information will soon be published. The article is still within publication process (Mehling et al. submitted to Forensic Sci Int "*Determination of GHB and GHB-O-β-glucuronide in hair of three narcoleptic patients - Comparison between single and chronic GHB exposure"*).

3.2. Biochemical investigations

The previously published results showed that the determination of the isotope ratio of GHB after GHB intake enables to distinguish between endogenously produced and exogenous ingested GHB [16]. Firstly the Carbon isotope ratio (CIR) of Xyrem® was measured and correlated to the internationalen Standard Vienna Pee Dee Belemnite (VPDB): $\delta^{13}C_{VPDB}$ = -28.6±0.1 ‰ [16]. Afterwards the CIR $\delta^{13}C_{VPDB}$ were determined in the collected urine samples (Tab. 1).

Tab. 1. Concentrations and CIR of GHB in urine samples, CIR = carbon isotope ratio.

Time [h]	Concentration [mg/L]	CIR (GHB)
0	1.69	-23.66
2	254	-28.66
4	66.9	-28.42
6	4.25	-25.72
8	1.56	-24.11
12	1.55	-24.29
22	0.83	not detected
46	0.88	not detected
70	0.91	not detected

The results show that the CIR values of urinary GHB are significantly influenced after administration. Presumably due to its fast metabolism, differences in the isotope composition of the analyzed molecules could only be detected up to 4 hours after GHB intake. Therefore, the detection window of GHB intake could not be prolonged by using this technique.

3.3. Genetic Investigations

We investigated the expression of *ALDH5A1*, *AKR7A2*, *EREG* and *PEA-15* of P1, P2 and P3 after GHB intake and compared the results with the gene expression levels of the control group. The genes of interest *ALDH5A1*, *AKR7A2*, *EREG* and *PEA15* showed no alterations in gene expression compared to the values of the control group. Further information are shown in a recently published article in Int J LegMed (Mehling et al. Int J Legal Med "Alterations in gene expression after gamma-hydroxybutyric acid intake - a pilot study*", accepted may 2017*).

4. Conclusions

In this study interdisciplinary investigations were performed to find a potential biomarker or a new strategy for the identification of a GHB intake. In summary, no promising approach could reach this aim neither toxicological, biochemical nor genetic investigations. The metabolites GHB-Gluc and GHB-Sulf could not widen the detection window in plasma and urine samples. Furthermore, hair analysis could not provide an evidence for single or multiple GHB administrations in therapeutically dosages. Additionally, the isotope ratios of endogenously generated and industrially synthesized GHB could identify the origin of the excreted GHB in urine samples after ingestion, but this approach could not extend the detection window. Finally, no significant differences in expression of the genes *ALDH5A1*, *AKR7A2*, *EREG* and *PEA15* between peripheral blood samples in case and control groups were observed. Further investigations should be conducted to find another approach to extend the detection window after GHB intake.

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