Phase I metabolites (organic acids) of gamma-hydroxybutyric acid—validated quantification using GC–MS and description of endogenous concentration ranges

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Abstract
Gamma-hydroxybutyric acid (GHB) is a sedative drug used in drug-facilitated crimes. Its detection window is very short. GHB undergoes intensive phase I metabolism to organic acids (glycolic acid, succinic acid, dihydroxybutyric acids). These could be potential analytical targets to broaden the detection window. The aim of the present study was to enable the detection of endogenous levels of these metabolites in biological samples (blood and urine). A gas chromatographic–mass spectrometric method using liquid–liquid extraction and derivatization with N-methyl-N-trimethylsilyltrifluoracetamide was developed for the quantification. Validation results were consistent with international guidelines, and the method was able to quantify endogenous levels of the substances in both urine and blood. Endogenous concentrations were shown to be <0.03–4.92 mg/L for glycolic acid, <0.03–1.28 mg/L for GHB, <0.28–18.1 mg/L for succinic acid, <0.12–1.38 mg/L for 2,4-dihydroxybutyric acid, and <0.13–2.59 mg/L for 3,4-dihydroxybutyric acid in serum samples of 101 volunteers. Urinary endogenous concentrations were shown to be 1.30–400 mg/L for glycolic acid, <0.03–1.94 mg/L for GHB, 1.17–2.73 mg/L for succinic acid, 0.72–26.2 mg/L for 2,4-dihydroxybutyric acid, and 1.88–122 mg/L for 3,4-dihydroxybutyric acid in urine samples of 132 volunteers. These endogenous concentrations represent a basis to which concentrations after the intake of GHB can be compared to in order to prove the intake of this substance.

KEYWORDS
detection window, dihydroxybutyric acid, endogenous concentrations, gamma-hydroxybutyric acid, gas chromatography–mass spectrometry

1 | INTRODUCTION

Gamma-hydroxybutyric acid (GHB), a low-molecular-weight substance, is a short-chain fatty acid that is naturally found in most mammalian species.1,2 GHB is an endogenous substance produced through the degradation of the inhibitory neurotransmitter gamma-aminobutyric acid; so far, its natural function has not been fully elucidated.3 GHB is a central nervous system depressant capable of producing heavy sedative effects.4–6 GHB was used as an anesthetic in the early 1960s,7,8 but its application was stopped because of its adverse effects.9 It is also used to treat alcoholism and opiate withdrawal syndrome.10,11 It is
now widely used in the treatment of sleep disorders. However, in nontherapeutic use, GHB is frequently used by youth and bodybuilders or as a weight-loss supplement.

The effects of GHB include sedation, drowsiness, forgetfulness, and muscle relaxation. In addition, because it is colorless and nearly odorless, its detection is difficult, and thus, it is used as a knockout drug in cases of drug-facilitated sexual assaults. Therefore, GHB is currently a highly controlled substance worldwide, and its possession, sale, import, and export are prohibited.

Because GHB is an endogenous substance, it is important to distinguish the uptake from endogenous concentrations in biological samples in forensic cases. However, because of the relatively short half-life, detection windows in blood and urine are maximum 6 and 12 h, respectively. In addition, the lack of useful metabolites as biomarkers leads to difficulties in detection of this substance.

Recent results from measuring concentrations of both GHB phase II metabolites, GHB-glucuronide and GHB-4-sulfate, indicate that these markers are not reliable and appropriate for expanding the detection window after GHB use or forensic toxicology.

GHB is quickly metabolized to succinic semialdehyde by gamma-hydroxybutyric acid-dehydrogenase and to succinic acid, "a mediator of the citric acid cycle," by succinic semialdehyde dehydrogenase. It has been observed that the levels of the number of alpha and beta metabolites of GHB increase after GHB consumption, namely, organic acids 2,4-dihydroxybutyric acid, 3,4-dihydroxybutyric acid, and 4,5-dihydroxyhexanoic acid that have been described as part of phase I GHB metabolism. Chemical structures of these organic acids are shown in Table 1.

In clinical laboratories, GHB metabolites are already used for the diagnosis of hereditary illnesses like fumarase deficiency, succinate semialdehyde dehydrogenase deficiency (succinic acid), or primary hyperoxaluria type II (glycolic acid). Overall, approximately 50 diseases have been described in which an inherited single-enzyme defect causes a high concentration of acidic metabolites in the blood or urine. However, the determination of glycolic acid can also have a value in ethylene glycol poisonings.

Currently, the phase I metabolites of GHB have not been quantitatively determined in blood and urine samples in forensic laboratories. The aim of the present study was to enable the detection of these metabolites in biological samples and to describe endogenous reference ranges. Therefore, a routine method for the quantification of GHB and its isomers 2-hydroxybutyrate, 3-hydroxybutyrate, and 3-hydroxyisobutyrate, and of organic acids within GHB metabolism (glycolic acid, succinic acid, 2,4-dihydroxybutyrate, and 3,4-dihydroxybutyrate) using gas chromatography–mass spectrometry (GC–MS) had to be developed and validated.

### TABLE 1  Chemical structures of analyzed organic acids

| Substance                     | Chemical structures                                      |
|-------------------------------|----------------------------------------------------------|
| Gamma-hydroxybutyric acid     | ![Gamma-hydroxybutyric acid](image1)                      |
| Glycolic acid                 | ![Glycolic acid](image2)                                  |
| Succinic acid                 | ![Succinic acid](image3)                                  |
| 2,4-Dihydroxybutyric acid     | ![2,4-Dihydroxybutyric acid](image4)                     |
| 3,4-Dihydroxybutyric acid     | ![3,4-Dihydroxybutyric acid](image5)                     |

2. **MATERIALS AND METHODS**

#### 2.1  Chemicals and reagents

GHB sodium salt, glycolic acid, 2-hydroxybutyrate (alpha-hydroxybutyric acid) and 3-hydroxybutyrate (beta-hydroxybutyric acid), 3-hydroxyisobutyrate, succinic acid, 2,4-dihydroxybutyrate, and 3,4-dihydroxybutyrate were provided by Sigma-Aldrich (Taufkirchen, Germany). All standard stock solutions (10 mg/mL) were prepared using methanol.

The deuterated internal standard (GHB-d$_6$, 1.0 mg/mL in methanol) was purchased from Cerilliant (Round Rock, Texas, USA) and was diluted with methanol to obtain a stock solution of 500 µg/mL. N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) was purchased from CS-Chromatographie Service GmbH (Langenwehe, Germany). Hydrochloric acid was purchased from J. T. Baker (Deventer, The Netherlands), hydrochloric acid 0.1 mol/L in 2-propanol from Merck (Darmstadt, Germany), and ethyl acetate from Fisher Scientific (Loughborough, UK). All chemicals used in the present study were of analytical grade and were stored according to the instructions provided by the supplier.
2.2 Biological material for control samples

It is not possible to obtain blank serum and urine because all of the analytes detected here are of endogenous origin. Serum and urine samples were provided by six different patients referred to the MVZ Dr. Eberhard & Partner in Dortmund, Germany, and were used for selectivity studies and production of spiked serum and urine samples for validation. To avoid bacteria contamination in the urine samples, two to three droplets of chloroform were added, and all samples were stored at −20°C until further use.

2.3 Liquid–liquid extraction

Urine or serum (1 mL) was added to 40 μL of the internal standard GHB-d₆ (500 μg/mL). An amount of 100 μL of 6M hydrochloric acid was added to adjust the pH to 1–2. Finally, the analytes were extracted using 5 mL of ethyl acetate. The mixture was thoroughly shaken for 1 min and centrifuged at 3000 rpm for 3 min. The supernatant was carefully transferred to another glass test tube. Then, 100 μL of HCl (0.1 mol/L in 2-propanol) was added, and the supernatant was dried under a gentle stream of nitrogen at room temperature. The dried residue was derivatized and trimethylsilylated using 500 μL of MSTFA at 60°C for 60 min. The reaction mixture was transferred to an autosampler vial, and 2 μL was injected into the GC–MS system.

2.4 GC–MS conditions

GC–MS was accomplished using an MSD 5975C (Agilent Technologies, Santa Clara, USA) equipped with a 7890A GC (Agilent) and a 7683B autosampler (Agilent). The capillary column used was a TG-5SILMS (30 m × 0.25 mm i.d., 0.25-μm film thickness) (Thermo Fisher Scientific, Waltham, MA, USA). The GC was operated in the split mode (1/20 split ratio), and the injector and detector temperatures were set at 280°C and 230°C, respectively. The temperature program used was as follows: an increase of 10°C/min from 80°C to 300°C, with the initial temperature maintained for 2 min and the final temperature maintained for 3 min. High-purity helium was used as the carrier gas at a flow rate of 0.9 mL/min.

The compounds were ionized by an electron impact of 70 eV. The mass selective detector was used in selected ion monitoring mode for quantitative analysis. The following ions were monitored (using a dwell time of 20 ms): GHB: m/z 233 (target), 204; GHB-d₆: m/z 239 (target), 206; glycolic acid: m/z 205 (target), 147; 2-hydroxybutyrate: m/z 131 (target), 190; 3-hydroxybutyrate: m/z 191 (target), 192, 204; 3-hydroxyisobutyrate: m/z 177 (target), 218; succinic acid: m/z 247 (target), 218, 262; 2,4-dihydroxybutyrate: m/z 219 (target), 321; and 3,4-dihydroxybutyrate: m/z 233 (target), 321.

2.5 Assay validation for serum and urine analyses

The GC–MS procedure was validated for the quantification of the aforementioned organic acids in accordance with an international guideline. For drawing the calibration curves and for performing the quantitative measurements, the ratios of the peak area of the target ion of the organic acids to the peak area of the internal standard GHB-d₆ were applied.

The linearity and sensitivity of the method were tested in water. The comparison of calibration curves within the matrix water and those within the matrices serum or urine revealed the validity of the matrix water (comparable incline of the calibration curves). The method’s selectivity and specificity, accuracy, precision, and stability of the analytes were evaluated in both serum and urine.

2.5.1 Selectivity

Because every single analyte detected by the described method is endogenously present in serum and urine samples, usual selectivity studies are not possible. Nevertheless, six different sources of serum and urine samples were analyzed for peaks interfering (peak shoulders, wrong ratio of peak area of the target to peak area of the qualifier ion) with the signals of the analytes or the internal standard.

2.5.2 Linearity

Linearity was studied by analyzing a seven-point calibration using the concentrations of 1, 2.5, 5, 7.5, 10, 15, and 20 mg/L of all analytes in water. The curve was created six times on different days.

2.5.3 Accuracy and precision

Quality control samples were prepared using pooled blank serum or urine and spiked to provide two final concentrations (~2 and ~17 mg/L for each analyte). The quality controls of each concentration were measured twice on eight consecutive days. The concentrations of the analytes were assayed against a linear regression model. The program Valistat (version 2.0, Arvecon, Walldorf, Germany) was used. The calculated values at each concentration were averaged, and the percentage bias was calculated to estimate accuracy. The intra-and inter-day precision (relative standard deviation) was assessed from the comparison of the analysis of two control samples at each concentration on eight consecutive days. One-way ANOVA was used for precision calculations.

2.5.4 Limits of the method

The limit of detection (LoD) and limit of quantification (LoQ) were determined using a calibration curve in the low-concentration range according to DIN 32646. A seven-point calibration using the concentrations of 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mg/L of all analytes in water was recorded. Only those calibration points where S/N ratio was >3 were used (minimum five calibration points).
2.5.5 | Stability

Stability was tested in the extracted samples within the autosampler (at room temperature) and in serum and urine samples.

The stability of the extracts within the autosampler was evaluated for 40 h after extraction. Six control samples each for low (2 mg/L) and high (17 mg/L) concentrations were extracted, connected, and aliquoted again into six extracts. These extracts were injected into the device at 0, 4, 10, 15, 24, and 40 h after extraction and derivatization and were analyzed using the validated method.

In a second step, the stability of the analytes in serum and urine samples was tested by storing 20 measured real serum and urine samples at −20°C. After 1 month, the samples were reextracted, analytes were reexamined, and the observed results were compared.

2.6 | Detection of endogenous concentrations

After the method was validated, it was used to determine the endogenous levels of the organic acids: GHB, succinic acid, glycolic acid, 2,4-dihydroxybutyric acid, and 3,4-dihydroxybutyric acid. Authentic serum (n = 101) and urine (n = 132) samples taken from a mixed population (serum: 55 men, 46 women; urine: 77 men, 55 women) were evaluated. The intake of GHB or related substances was excluded among these patients using a questionnaire after informed consent. After the samples were received, the serum was quickly separated from the red blood cells, and serum and urine samples were stored at −20°C until further analysis, which was a maximum of 7 days after blood or urine sampling.

3 | RESULTS AND DISCUSSION

This method was specifically designed for the quantitative evaluation of GHB and endogenous organic acids within GHB phase I metabolism in serum and urine samples. The method involves an easy liquid–liquid extraction step and then derivatization using MSTFA and gas chromatographic mass spectrometric detection in selected ion monitoring mode. The important analytes to quantify for forensic purposes were GHB, glycolic acid, succinic acid, 2,4-dihydroxybutyric acid, and 3,4-dihydroxybutyric acid. However, GHB isomers—2-hydroxybutyrate, 3-hydroxybutyrate, and 3-hydroxyisobutyrate—were also included in the method to show chromatographic separation of the isomers and no interference of these substances with GHB detection. A chromatogram of the ions of all analytes within a quality control sample is shown in Figure 1. No peak shoulders or wrong ion ratios (compared to methanolic solutions) were detected when urine or serum samples were analyzed for selectivity studies.

The method was validated according to the international guideline. Accordingly, the linearity, LoD and LoQ, intra- and interassay precision, accuracy, stability in the extract, and long-term stability in serum and urine samples were evaluated. The statistical analysis of the results showed linearity of all analytes in the range 1–20 mg/L. The linear regression showed correlation coefficients always as $R^2 > 0.99$. Linearity and variance homogeneity were demonstrated within the working range using Fisher’s test (significant 99%) and Cochran’s test (significant 99%), respectively. Weighing of the calibration curves was not necessary. The concentration of 20 mg/L was selected as the highest calibrator. For none of the analytes real serum sample analytes with concentrations higher than 20 mg/L were detected. Therefore, the linearity range was considered to be the

![Figure 1](https://wileyonlinelibrary.com)
concentration range detected in real serum samples. However, urine samples showed concentrations >20 mg/L for some analytes. In these cases, samples had to be diluted before extraction. The method's imprecision and bias were always less than 15% and compliant with the guideline. Bias and precision data were reported in RSD% and are presented in Table 2.

The analytical limits of the method determined by a calibration curve in the low-concentration range according to DIN 32646 are shown in Table 3. The method showed limits of detection that were <0.3 mg/L and limits of quantification that were <1 mg/L for all analytes. Because most of the analytes could be detected at concentrations >1 mg/L (however, for GHB only 36.3% of the cases in urine and 0.9% of the cases in serum were >1 mg/L; for 2,4-dihydroxybutyric acid, basic concentrations in serum were mostly ~1 mg/L, in urine higher), the sensitivity of the method is considered to be sufficient. Regarding GHB, the analytical methods published23,36 showed similar or higher analytical limits.

The stability of the derivatized analytes was determined at both high (17 mg/L) and low (2 mg/L) concentrations so that the extracted sample was injected into the device six times consecutively over a time period of 40 h. The decline of the detected peak areas was less than 10% for all analytes. Therefore, the stability of the derivatized substances within the extracts was considered to be acceptable.

The stability of these substances in serum or urine samples for forensic studies could be a major problem because all of the substances are of endogenous origin, and for some analytes, some pathways can lead to biosynthesis or degradation. To determine the stability and variation extent of the analytes in serum and urine samples, 20 serum and urine samples were collected from the laboratory directly and, after the initial measurements, the parameters were remeasured 1 month after the first measurement.

It is well known that GHB concentrations can change during storage. Results of some studies, however, differ. Blood samples stabilized with NaF (n = 27 living individuals) were stored at −20°C and measured for GHB. Long-term stability could be demonstrated over a period of 7 years, and the deviation to the primary concentrations ranged between −32.4% and 21.0%. A good stability could also be demonstrated for serum samples without stabilization. Samples were stored for 9 months at −20°C and measured monthly in triplicates. In addition, stability at room temperature was retained over the test period of 48 h (maximum deviation 11%). This provides the possibility of maintaining stability during postal transfer between the police department and analytical laboratory. Three freeze–thaw cycles did not have an influence on the measured GHB concentrations (maximum deviation 11%). Jones et al.39 investigated the stability of 50 whole blood samples from impaired drivers, stored at 4°C for up to 1 year. The results obtained indicated that blood GHB levels are stable when stored at 4°C for up to 6 months. Beránková et al.40 studied the GHB stability in blood and urine with and without sodium fluoride (1% w/v) at 4°C and −20°C for up to 8 months. Ante-mortem samples showed no significant GHB production.

However, recently a study showed the instability of GHB in serum samples: Busardo et al.41 determined the stability at −20°C, 4°C, and 20°C. Blood samples showed a decrease in GHB levels only after 3 days of storage at −20°C and at +4°C (decrease was always less than 10%; samples stored at +20°C showed a mean decrease of 10.4%). After 4 weeks of storage, the mean decrease in GHB concentrations was higher than 20% at all storage temperatures. Urine samples showed a decrease in GHB levels higher than 10% after just 3 days of storage for samples kept at all tested temperatures. After 4 weeks of storage, the mean decrease in GHB

| TABLE 2 | Bias and precision data of the analytical method |
|----------|-----------------------------------------------|
| Substance | Concentration (mg/L) | Bias (%) | Intra-day precision (%) | Inter-day precision (%) |
| Gamma-hydroxybutyric acid | 2 | −4.3 | 6.6 | 7.8 |
| | 17 | 0.82 | 2.5 | 3.8 |
| Glycolic acid | 2 | 0.90 | 6.6 | 11.0 |
| | 17 | 0.39 | 1.4 | 5.7 |
| Succinic acid | 2 | −8.5 | 8.3 | 10.4 |
| | 17 | 0.81 | 3.7 | 4.9 |
| 2,4-Dihydroxybutyric acid | 2 | 5.7 | 7.1 | 7.1 |
| | 17 | −1.3 | 5.3 | 5.2 |
| 3,4-Dihydroxybutyric acid | 2 | 13.0 | 1.8 | 2.5 |
| | 17 | −0.36 | 3.1 | 4.2 |

| TABLE 3 | Analytical limits of the method |
|----------|--------------------------------|
| Substance | Limit of detection (mg/L) | Limit of quantification (mg/L) |
| Gamma-hydroxybutyric acid | 0.03 | 0.11 |
| Glycolic acid | 0.19 | 0.63 |
| Succinic acid | 0.28 | 0.98 |
| 2,4-Dihydroxybutyric acid | 0.12 | 0.39 |
| 3,4-Dihydroxybutyric acid | 0.13 | 0.42 |
concentrations was higher than 25% at all storage temperatures. According to their findings, Busardo et al. recommended the analysis of GHB in blood and urine within 3 days of sampling and the storage at –20°C or 4°C to avoid instability issues. During validation experiments, we tested GHB stability at –20°C for 1 month. GHB concentrations were always less than LoQ in serum. In urine, the mean deviation from the first measurement was -9.6% (-27% to +5.6%).

The hydroxylated metabolites of GHB seemed to be very stable and did not change a lot after storage at –20°C for a month. 2,4-Dihydroxybutyric acid concentrations showed little deviation from the first measurement in serum (mean: -0.6%, range: -10% to +9.6%) and in urine (mean: -10%, range: -26% to +4.2%). 3,4-Dihydroxybutyric acid concentrations showed little deviation from the first measurement in serum (mean: -2.7%, range: -17% to +14%) and in urine (mean: -10%, range: -26% to +4.2%). Glyceric acid concentrations decreased with a mean of 16% (decrease of 11%-28%) in serum and with a mean of 11% (decrease of 28% to an increase of +25%) in urine samples. These data are within the accuracy of the method and can be considered to be acceptable. Therefore, it is assumed that 2,4-dihydroxybutyric acid, 3,4-dihydroxybutyric acid, and glyceric acid concentrations are stable up to 1 month when stored at –20°C.

In contrast, the concentrations of succinic acid changed considerably during storage. Within serum samples, the concentrations increased with a mean of 190% (decrease of 2% to an increase of 716%). In urine, an increase or decrease could be observed (mean: decrease of 11%; minimum –91%, maximum +52%). Therefore, it is assumed that succinic acid concentrations are very unstable, and it is recommended to measure succinic acid right after sampling. Concentrations measured in samples stored for some time even at –20°C are not usable.

Overall, the validation showed suitable selectivity, sensitivity, accuracy, precision, and linearity, which were in the range from the LoQ to 20 mg/L; all the mentioned parameters indicate the reproducibility and repeatability of this method and its suitability for quantitative analysis. The stability of succinic acid in serum as well as in urine samples seemed to be a problem; however, the stability of all other analytes over 1 month at –20°C was acceptable.

To confirm the applicability of the method and to determine endogenous concentrations of the parameters in human serum and urine, the levels of GHB, 3,4-dihydroxybutyrate, 2,4-dihydroxybutyrate, succinic acid, and glyceric acid were measured using the validated method in the collective. Table 4 presents the minima, maxima, median, and mean values of the concentrations of the organic acids detected in serum samples (n = 101). Table 5 presents the minima, maxima, median, and mean values of the concentrations of the organic acids detected in urine samples (n = 132).

As other studies have shown, GHB could be detected at concentrations <1.28 mg/L in serum and <2 mg/L in urine. Elian showed endogenous GHB concentrations in 240 blood (0.17–1.51 mg/L) and 670 urine samples (0.34–5.75 mg/L). Andresen et al. showed plasma concentrations of 0.62–3.2 mg/L (n = 50) and urine concentrations of 0.64–4.2 mg/L (n = 50). Erdmann et al. showed blood concentrations of 0.11–1.56 mg/L. Elliot analyzed 119 urine samples and determined the endogenous concentrations to be <3 mg/L. Kang et al. showed urinary concentrations between 0.09 and 1.8 mg/L in 74 subjects, whereas Brailsford et al. determined GHB in urine of 1126 females and showed concentrations of up to 5.5 mg/L. Because LeBeau et al. had shown that GHB can also be built during storage, the recommendations for endogenous/exogenous cutoffs are <4 μg/mL in serum and <6 μg/mL in urine.

Phase II metabolites of GHB have been described (GHB-O-glucuronid and GHB-4-sulfate in Petersen et al. and Shima et al., respectively); however, they could not be detected in higher concentrations after the intake of GHB. Therefore, the aim of our research was to determine if phase I metabolites of GHB can be

### TABLE 4  Mean endogenous serum concentrations of the analytes in the collective (n = 101)

|                          | Mean | Median | Maximum | Minimum         | Standard deviation |
|--------------------------|------|--------|---------|-----------------|--------------------|
| Gamma-hydroxybutyric acid (mg/L) | <0.11 | <0.11  | 1.28    | Not detected    | 0.17               |
| Glycolic acid (mg/L)     | 1.28 | 1.33   | 4.92    | 0.39            | 0.46               |
| Succinic acid (mg/L)     | 2.66 | 2.75   | 18.14   | Not detected    | 2.30               |
| 2,4-Dihydroxybutyric acid (mg/L) | 0.95 | 0.92   | 1.38    | Not detected    | 0.14               |
| Standard deviation:      | 1.33 | 1.27   | 2.59    | Not detected    | 0.33               |

### TABLE 5  Mean endogenous urine concentrations of the analytes in the collective (n = 132)

|                          | Mean  | Median | Maximum | Minimum         | Standard deviation |
|--------------------------|-------|--------|---------|-----------------|--------------------|
| Gamma-hydroxybutyric acid (mg/L) | 0.40  | 0.35   | 1.94    | Not detected    | 0.37               |
| Glycolic acid (mg/L)     | 46.0  | 26.8   | 400     | 1.30            | 64.0               |
| Succinic acid (mg/L)     | 27.0  | 15.6   | 273     | 1.17            | 35.8               |
| 2,4-Dihydroxybutyric acid (mg/L) | 5.25  | 4.35   | 26.2    | 0.72            | 4.09               |
| Standard deviation:      | 21.6  | 13.4   | 122     | 1.88            | 21.4               |
detected in higher concentrations after the uptake of GHB and if elevated concentrations above the endogenous level can be detected longer for the metabolites than for GHB itself. For this aim, a reference range for endogenous concentrations of these substances has to be established in typical forensic body fluids such as blood and urine.

Some organic acids have been described to be part of GHB metabolism. The incorporation into succinic acid of label from [1-14C]- and [4-14C]-GHB given intravenously or intraperitoneally to rats and cats accounts for only a small proportion of the metabolized labeled substances has to be established in typical forensic body fluids such as blood and urine.

Palomino-Schätzlein et al.55 searched for metabolites associated with GHB consumption using a metabolomics approach by nuclear magnetic resonance spectroscopy. The results showed that concentrations of glycolate and succinate increased after 1 h of drug ingestion. Twelve volunteers consumed 25 mg GHB/kg body weight. Whereas GHB and succinate concentrations decreased rapidly to an endogenous level (at 6 h) which was, however, not quantified exactly, glycolate concentration decreased slowly, and even after 24 h, a small difference could be observed. Steurer et al.56 identified small but significant differences in glycolate levels in urine samples taken 4 h after the intake of 50 mg/kg body weight in an untargeted metabolomics approach. However, a quantification of these substances and a description of their endogenous concentration range have, to the best of our knowledge, never been conducted at least in forensic laboratories.

We concentrated on glycolic acid, succinic acid, 2,4-dihydroxybutyric acid, and 3,4 hydroxybutyric acid because these substances are commercially available and could be potential target analytes after the intake of GHB.

In our study, 2,4-dihydroxybutyrate and 3,4-dihydroxybutyrate could be detected in serum at concentrations ranging from less than LoD to 1.38 mg/L and less than LoD to 2.59 mg/L, respectively. It could be confirmed that β-oxidation of GHB is preferred over α-oxidation.29 Within urine samples, concentrations were clearly higher (0.72–26.3 and 1.88–122 mg/L, respectively). The results of a Shapiro–Wilk test indicated that the distribution of data in urine and serum for both parameters was not normal (P < 0.05). Fell et al.57 had also determined 3,4-dihydroxybutyrate concentrations in urine of 21 adults and showed a range of 23–104 mg per 24 h. Serum concentrations of 3,4-dihydroxybutyrate were also reported for four samples in the range of 0.144–0.264 mg/L. Moreover, Bouatra et al.58 conducted a study on human metabolites in the urine of 22 healthy adult volunteers and found that the concentrations of 3,4-dihydroxybutyrate and 2,4-dihydroxybutyrate were 21.9–56.1 μmol/mmol creatinine and 0.3–1.8 μmol/mmol creatinine, respectively. The results of these studies were consistent with those determined in our study. However, Keyfi et al.33 determined 2,4-dihydroxybutyric acid and 3,4-dihydroxybutyric acid concentrations in the urine of 251 healthy volunteers and defined reference ranges with <4 mmol/mol creatinine for 2,4-dihydroxybutyric acid and <7mmol/mol creatinine for 3,4-dihydroxybutyric acid. In our study, the mean values were 13 mmol/mol creatinine and 47 mmol/mol creatinine and were clearly higher than those within this reference population in Iran.33 However, Keyfi et al.33 did not provide any details on the method to quantify organic acids and on the method validation. Furthermore, Zhou et al.59 have shown that increased urinary 3,4-dihydroxybutyrate and 2,4-dihydroxybutyrate levels may be associated with bladder cancer.

The endogenous concentrations of succinic acid in our study were less than LoD to 18.1 mg/L in serum and 1.17–273 mg/L in urine. Nordmann and Nordmann had shown concentrations of 0.05–0.07 mg/L in blood, and 2–12 mg of succinic acid were excreted in the urine over 24 h.60 Asano et al. showed a maximum urinary concentration of 3.7 mg/L (n = 5).61 Keyfi et al. showed urinary concentrations in 251 healthy individuals and defined a reference range of <200 mmol/mol creatinine in urine.33 In our study the mean concentration in urine was 66 mmol/mol creatinine; however, there were some patients with higher urinary concentrations of succinic acid. However, the instability of succinic acid in stored samples must be considered. Within our study, samples were always measured within 1 week after sampling.

Glycolid acid could be detected at concentrations of 0.39–4.92 mg/L in serum and 1.3–400 mg/L in urine. Keyfi et al.33 defined the reference range of urinary concentrations in 251 healthy individuals to be <16 mmol/mol creatinine in urine. In our study, the mean concentration of glycolic acid in urine was 104 mmol/mol creatinine, and many patients had higher concentrations than the reference range defined by Keyfi et al. Again it should be mentioned that Keyfi et al.33 did not provide any details on the method to quantify organic acids and on the validation data of the method.
4 | CONCLUSIONS

A method was developed and validated to quantify GHB and its phase I metabolites, namely succinic acid, glycolic acid, 2,4-dihydroxybutyric acid, and 3,4 dihydroxybutyric acid, in human serum and urine. The endogenous concentration ranges for these analytes in urine and serum were defined. In a future study, these metabolites must be determined in samples of patients who chronically or uniquely take GHB for medical reasons or in forensic cases after the known intake of GHB to evaluate if these parameters could be useful biomarkers with regard to the detection of a GHB intake.

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