Introduction

Although gamma-hydroxybutyric acid (GHB) is used as a pharmaceutical drug in the treatment of narcolepsy and cataplexy, illicit GHB continues to be abused, with numerous reports on acute poisoning, complicated withdrawal symptoms and delirium, drug-related deaths and potential use in drug-facilitated sexual assault (1–5). The consumption of GHB or its chemical precursors 1,4-butanediol and gamma-butyrolactone (GBL), can cause serious toxic effects, including impaired consciousness and coma. Because of the fast metabolization of GHB via the Krebs cycle, less than 1% of the drug is excreted unchanged in urine. After the consumption of a pharmaceutically active single dose, GHB is only detectable above the currently accepted cutoff value (10 μg/mL) in a narrow time window of 3–6 h (6, 7).

An enzymatic method for GHB detection has recently been reported, but no immunoassay screening kits are available due to the uncharacteristic structure of GHB (8). The analysis of GHB has previously been performed by laborious methods such as gas chromatography and mass spectrometry (GC–MS) after liquid–liquid extraction and conversion of GHB into GBL (9) or after derivatization with silylation agents (10). However, it is more convenient to use liquid chromatography and tandem mass spectrometry (LC–MS–MS) for the routine quantification of GHB in serum, urine and hair (11, 12). Nevertheless, current analytical methods only determine unchanged GHB excreted in urine; the interpretation of data continues to be problematic regarding the discrimination between endogenous levels and exogenous ingestion. Consequently, any analytical method that can extend the detection window for the ingestion of GHB or lower the cutoff values to discriminate endogenous GHB from the administered drug will represent an important advance in clinical and forensic toxicology.

The aim of the present study was to investigate the existence in vivo of an O-gluconoride of GHB by analogy with ethyl glucuronide, which has been recognized as an excellent biomarker for alcohol consumption due to its longer half-life in plasma than ethanol (13). Thus far, no conjugation studies of GHB have ever been published. A compound like GHB, with both hydroxy- and carboxy-group functionality, should theoretically be an appropriate substrate for the enzymatic glucuronidation by UDP-glucuronosyltransferase (14).

To test this hypothesis, reference standards of the O-glucuronide derivative of GHB (GHB-GLUC) and its deuterium-labeled internal standard (IS; d4-GHB-GLUC) were synthesized (15). The structures of both analytes are shown in Figure 1. Targeted analysis of urine samples was performed using a newly developed LC–MS–MS method. The bioanalytical results are presented in the following, with a discussion of the future implications and studies needed to fully evaluate GHB-GLUC as a potential biomarker for GHB ingestion and intoxication. The method for chemical synthesis has recently been published with all details (including a stability study) in the organic chemistry literature (15).

Experimental

Chemicals

GHB-GLUC (β-O-gluconoride of GHB) and the corresponding deuterium-labeled analogue, d4-GHB-GLUC, were synthesized at the Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen (15). Using high-resolution MS at an external laboratory (data not shown), the exact masses were found to be within 5 ppm of theoretical values. Impurity testing of d4-GHB-GLUC showed less than 0.14% contents of GHB-GLUC, which was found to be acceptable for using the compound as an internal chromatographic standard. Stock solutions (1 mg/mL) of GHB-GLUC and d4-GHB-GLUC were prepared in purified water and stored in the dark at +4 °C. Organic solvents and reagents were of analytical grade. Purified water (18 MΩ) was generated in an Elga Centra RDS system (Buckinghamshire, UK).

Methods

LC–MS–MS

The LC–MS–MS system was a 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA) with a
Jetstream electrospray ion source operated at unit resolution. The chromatographic system included an Agilent 1200 binary pump, a 1200 SL autosampler and an 1100 column department. Separation was performed on an XDB-C18 column (50 × 4.6 mm) with particle size of 1.8 μm (Agilent Technologies). The flow rate was 600 μL/min using the following mobile phases: 0.4% formic acid in purified water (A) and acetonitrile (B). Gradient elution was as follows: 0% B for 1.8 min, up to 90% B in 3.2 min, 90% B for 0.5 min, followed by equilibration at initial conditions for 3.5 min. The column temperature was 40°C. The autosampler injection volume was 10 μL. The total run time was 9 min.

Ion source parameters and individual collision energies (range: 10–22 V) were initially optimized in flow injection mode. The capillary voltage was 3,500 V, nitrogen flow was 7 L/min and nitrogen gas temperature was 350°C. The sheath gas flow was 8.5 L/min. The sheath gas temperature was 400°C and the nozzle voltage was 400 V in positive mode. The nebulizer pressure was 20 psi. The declustering potential was 110 V. Multiple reaction monitoring (MRM) with dwell time of 200 ms was performed by using the deprotonated molecule [M–H]− for GHB-GLUC (m/z 279) and six product ions (m/z 193, 103, 113, 85, 73, 71 and 59), and the deprotonated molecule for d5-GHB-GLUC (m/z 283) with three qualifying product ions (m/z 59, 73 and 85). The highest intensity transition for GHB-GLUC, m/z 279 → 113, was used for calibration and quantification. The urinary concentrations were determined from the peak area ratios of GHB-GLUC to endogenous concentration of d5-GHB-GLUC by reference to a calibration curve covering 0.1 to 10 μg/mL of GHB-GLUC. Data acquisition and analysis were performed with MassHunter software (Agilent Technologies).

The identification of GHB-GLUC in unknown urine samples was approved for correctly assigned retention times (tolerance ± 2.5%) and from MRM data, in which the relative ratios for six qualifying ions, expressed as a percentage of the intensity of the most intense transition, were within ± 20% of the mean values determined in calibrators with a signal-to-noise ratio (S/N) above 3. These performance criteria comply with a European Union (EU) directive concerning quality assurance for the reliable identification and confirmation of target compounds in biological samples (16).

**Calibration standards**

Urine calibrators (n = 7) were prepared by spiking appropriate aqueous working solutions of GHB-GLUC into a donor urine pool. The concentrations were 0.1, 0.25, 0.5, 1, 2, 5 and 10 μg/mL, plus the endogenous GHB-GLUC concentration (0.21 μg/mL), which was calculated by a single standard addition experiment as the X-intercept at Y = 0 from the linear regression calibration curve. To test for matrix effects, a series of aqueous calibrators (n = 7) were prepared by spiking appropriate aqueous working solutions of GHB-GLUC into purified water. The concentrations were 0.1, 0.25, 0.5, 1, 2, 5 and 10 μg/mL. Standard curves based on peak area with a linear curve fit (Y = ax² + b), not forced through zero and with no weighting, were used for calibration.

**Urine samples**

Endogenous concentrations were determined in anonymous clinical urine samples (n = 50). The samples were randomly selected from regional patients requiring general health screening and routine medical examination at the Department of Clinical Biochemistry, Vendsyssel Hospital. According to Danish Law [Number 593 of June 14, 2011, 14(3)] on scientific research, these fully anonymous materials can be studied without approval by the local Human Ethics Committee. All samples were kept at 4°C and analyzed for GHB-GLUC in a single batch. Additionally, GHB was analyzed with a routine LC–MS-MS method implemented following the method of Wood et al. (12) with a limit of quantification (LOQ) of 0.5 μg/mL. Creatinine in urine was assayed by using a routine clinical method based on the Jaffe reaction (Siemens Healthcare, Erlangen, Germany).

**Sample preparation**

Sample preparation was identical for calibrators, samples and controls. Before analysis, a fresh IS working solution was prepared by the dilution of 10 μL (1 mg/mL of d5-GHB-GLUC) into 100 mL of mobile phase A. Samples (100 μL) were mixed with 900 μL of IS working solution, transferred to vials and placed in the autosampler.

**Stability of GHB-GLUC toward acidic or alkaline hydrolysis**

The stability of GHB-GLUC under acidic and alkaline conditions was evaluated by autoclaving a 10 mg/mL aqueous calibration standard of GHB-GLUC in solutions of 4 M hydrochloric acid and 3 M sodium hydroxide for 15 min. The stability of d5-GHB-GLUC was not tested.

**Method performance**

Intra-assay method precision and accuracy were assessed by the analysis of spiked urine control samples (n = 5) in three concentrations (0.25, 1 and 5 μg/mL) plus endogenous concentration of 0.21 μg/mL. Results were corrected for the endogenous concentration, which was calculated by the standard addition method as the X-intercept at Y = 0 from the linear regression calibration curve. The same urine pool was used for the preparation of calibration standards and control samples. Precision was expressed as the relative standard deviation (RSD) and recovery was calculated as 100% × mean calculated concentration/spiked + endogenous concentration). Matrix effects were estimated by comparing ion intensities of aqueous calibration standards with equimolar urine.
Results

Mass spectral information
Electrospray mass ionization and detection of GHB is more sensitive in positive mode (þES), although the compound is a carboxylic acid, but the detection of GHB-GLUC is approximately six times more sensitive in negative electrospray ionization (−ES) than in þES. The −ES product ion mass spectra of deprotonated GHB-GLUC, m/z 279 and deprotonated d4-GHB-GLUC, m/z 283, show many prominent peaks that are suitable for MRM (Figure 2). However, most peaks originate from the electrophilic glucuronate moiety, except for m/z 103 and 107 in the product ion spectrum of d4-GHB-GLUC; the latter is the only prominent þ4 ion observed, containing the four deuterium atoms. However, m/z 103 must also partly originate from the glucuronate moiety because it is also a fragment of d4-GHB-GLUC. The ions m/z 75, 85, 95 and 113 have been reported in the product ion spectra of both ethyl glucuronide (EtG) and d5-EtG (17) and are part of a general fragmentation pattern observed for O-glucuronides (18).

Calibration and linearity
Because GHB and GHB-GLUC are naturally present in urine samples, low level calibrators cannot be prepared in a drug-free biological matrix. Consequently, the standard addition method was used to construct a calibration curve for GHB-GLUC in urine. Standard curves of both urine and aqueous calibrators showed linearity up to 10 µg/mL with correlation coefficients (r2) above 0.99. The slopes of the calibration lines in these two matrices were equal (e.g., water: 0.0011; urine: 0.0010), which indicates that a more simple approach using aqueous calibrators can be used in future studies or for improvement of the current method. However, because there is a general consensus in bioanalytical methods that calibrators should be prepared in the same biological matrix as the sample or one that matches the composition of the sample solution as closely as possible (16), the method presented here was validated according to this principle.

Precision and accuracy
The intra-assay precision (RSD) was 6.5 to 15% and recovery in the range 79–108%, which was considered to be acceptable for the purposes of the present study. The LOQ, for which all qualifying ion signals had S/N ≥ 3, was 0.1 µg/mL, which was estimated from samples with low endogenous concentrations. Below 0.1 µg/mL, GHB-GLUC could still be detected, but with a lower identification power because not all six qualifying ions could be detected. Thus, the limit of detection (LOD) for GHB-GLUC, using the MRM transition m/z 279 → 113, was 0.025 µg/mL. The LOD was also estimated from samples with low endogenous concentrations. The retention times varied

Figure 2. −ES product ion spectra of the deprotonated pseudomolecular ions: m/z 283, d4-GHB-GLUC (A); m/z 279, GHB-GLUC (B).
within the acceptance criterion; the worst case was +2.3% from the assigned value.

**Urine samples**

The results from targeted analysis of GHB-GLUC in urine samples are listed in Table I. All GHB concentrations were below the LOQ for this method (0.5 µg/mL). Thus, they were considered to be within the reference range (19). GHB-GLUC was detected in all samples, but three samples had concentrations below the LOQ. Concentrations were also calculated after normalization (correction) for urinary creatinine to express the concentration range free of influence from the variations in urine samples are listed in Table I. All GHB concentrations were below the LOQ for this method (0.5 µg/mL). Thus, they were considered to be within the reference range (19). GHB-GLUC was detected in all samples, but three samples had concentrations below the LOQ. Concentrations were also calculated after normalization (correction) for urinary creatinine to express the concentration range free of influence from the variations in

**Matrix effects**

To evaluate matrix effects (ion suppression) with the infusion method, extracts from blanks or pure matrix are normally injected into the LC system. Because true blank urine could not be provided for this purpose, matrix effects were estimated by comparing the ion intensities of aqueous calibration standard with equimolar urine or serum calibrators. The results showed 8–15% ion suppression, but this had no critical effect because d4-GHB-GLUC co-elutes with GHB-GLUC and compensates for the alteration in signal, thereby minimizing matrix and ion suppression effects on quantification results. Matrix effects from the dilute-and-shoot technique on the very polar GHB-GLUC compound may also explain the variation in retention times observed in the study.

**Effects of acidic or alkaline hydrolysis**

Under acidic conditions with autoclaving, GHB-GLUC was degraded completely; however, GHB-GLUC was stable under alkaline conditions with autoclaving.

**Discussion**

**Chemical synthesis**

Although a 95% pure di-sodium salt of GHB glucuronide (RCG-229) is commercially accessible in the 2012 catalogue from ReseChem GmbH (Burgdorf, Switzerland), a method for its chemical synthesis has not been published. At present, GHB-GLUC does not have a Chemical Abstract Services (CAS) number. No references to conjugates or glucuronides of GHB were found in the scientific literature, including the specific use of RCG-229. Because the use of an isotope labeled IS is essential in LC–MS-MS, the present study aimed at developing new synthetic methods to be used to provide highly pure reference compounds of both GHB-GLUC and d4-GHB-GLUC for future studies (15).

**Liquid chromatography**

Liquid chromatographic separation of GHB and GHB-GLUC was performed in gradient mode using a reversed-phase C18 column, which provides enhanced retention for carboxylic acids at low pH in the mobile phase. GHB-GLUC eluted as a symmetric peak with retention time of 3.1 min, as shown in the chromatogram of a urine sample (Figure 3).

**Glucuronidation**

The glucuronidation in vitro of small aliphatic alcohols and carboxylic acids is complex and not yet fully understood. The ether glucuronide of an alcohol differs in terms of stability in vivo. The glucuronidation of carboxylic acids is complex and not yet fully understood. The glucuronidation of an alcohol differs in terms of stability in vivo. The glucuronidation of carboxylic acids is complex and not yet fully understood. The ether glucuronide of an alcohol differs in terms of stability in vivo.
temperature, can undergo subsequent rearrangement processes into isomers that are resistant to enzymatic hydrolysis with beta-glucuronidase (21). Consequently, in the present study, only the ether \( \beta \)-O-glucuronide was synthesized, because this is the sole product formed \textit{in vivo} by UDP-glucuronosyltransferase (14).

Jurowitch \textit{et al.} studied the glucuronidation of aliphatic alcohols in human liver microsomes \textit{in vitro} and observed structure-related favoring of longer chain primary alcohols and mutual inhibition effects (22). Interestingly, ethanol, even in high concentrations, only slightly inhibited the glucuronidation of alcohols with a longer chain. Thus, the biosynthesis of a glucuronide of GHB \textit{in vivo} is theoretically feasible after the co-administration of GHB with alcohol.

**GHB-GLUC as a potential biomarker**

The most important prospect of GHB-GLUC is the potential to use the metabolite as a biomarker for GHB intoxication, by analogy with EtG, which is used as a routine biomarker for ethanol consumption (13), despite the discussions on false positive and false negative results (23, 24). However, to fully evaluate this possibility, detailed knowledge of the pharmacokinetic properties of GHB-GLUC must be available. Several conditions must be met or considered: (i) GHB-GLUC must have a longer plasma half-life than GHB with a concentration peaking later than 12 h; (ii) peak concentrations of GHB-GLUC must differ significantly from normal values; (iii) intra-individual and inter-individual variations of GHB-GLUC in normal, healthy individuals should be small or easy to distinguish from GHB-GLUC concentrations obtained after the administration of large doses of GHB; (iv) GHB-GLUC stability in the matrix is important.

There is a pressing need for clinical studies to fully assess and evaluate these preconditions for using GHB-GLUC as a biomarker in both urine and serum. The large inter-individual variation of GHB-GLUC concentrations observed in the present study of 50 clinical samples, even after adjustment for urine dilution (creatinine correction), does not look promising. If the absolute concentrations of GHB-GLUC are not applicable for the interpretation of GHB ingestion, then the relative concentration could be useful; e.g., in hair analysis in which a time profile can be established, provided that GHB-GLUC, like EtG, is incorporated into this sample matrix.

**Stability issues**

The present study shows that in some samples (e.g., Samples 43 and 47 in Table I), a large fraction (>90%) of GHB in urine can be found as the glucuronide. Although GHB-GLUC is stable in buffer solutions at pH 4.8 to 9 with temperatures from 18 to 90 °C (15) and stable during autoclaving under strong alkaline conditions, degradation by enzymes or other hydrolysis mechanisms in urine cannot be excluded.

LeBeau \textit{et al.} investigated variations in urinary concentrations of endogenous GHB (25, 26) and presented evidence for the \textit{in vitro} production of GHB in some samples in storage (27, 28). However, the endogenous production of GHB was also reported for blood samples (29, 30). Reports on these few, but highly elevated, GHB concentrations in biological samples have never been scientifically explained and studies into the subject do not consider the possible presence of GHB glucuronides or other conjugates. However, the observation of elevated GHB concentrations in a few samples has been the primary reason for implementing a relatively high cutoff value of 10 \( \mu \)g/mL GHB to avoid false positive results in testing for endogenous GHB.

The analytical procedure by LeBeau and other investigators included the treatment of the urine sample with concentrated sulfuric acid at 70–100 °C to convert all GHB to GBL before extraction and detection by GC–MS. In the development of a method for therapeutic drug monitoring, Ferrara \textit{et al.} described the optimization of reaction parameters (80°C, 20 min) for the conversion of GHB to GBL, which was performed to obtain the highest yield in blank urine samples (9). Under these conditions, a major part of the GHB-GLUC present in the samples must have been hydrolyzed, as was shown in the present study. Thus, it must be expected that population studies and reference ranges for GHB can be biased, with results depending on the experimental conditions and pre-treatment of the samples prior to detection. This may also explain that studies using GC–MS detection of GBL show a few samples with elevated levels; for example, in a population study by LeBeau \textit{et al.}, only five out of 207 individuals had elevated urine concentrations of GHB.
investigated in due course.

In hair analysis, for example, is promising and will be further evaluated. The prospects of using GHB-GLUC as a biomarker for the future analysis of GHB in clinical and forensic toxicology. The implications for the future analysis of GHB in clinical and forensic toxicology are promising.

This study has shown that GHB-GLUC is an important and hitherto unrecognized metabolite of GHB. This finding will have implications for the future analysis of GHB in clinical and forensic toxicology. The prospects of using GHB-GLUC as a biomarker in hair analysis, for example, is promising and will be further investigated in due course.

References
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