Intra- and Interindividual Variations in Urinary Concentrations of Endogenous Gamma-Hydroxybutyrate*

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Abstract

This study was designed to determine the urinary concentrations of endogenous GHB over a one-week period, the variations that occur within those concentrations, and whether those variations are affected by normalization to urinary creatinine. Its purpose was to ascertain whether endogenous concentrations fluctuate to such an extent that they may be misinterpreted as due to GHB ingestion. Every urine void produced by eight GHB-free subjects (five males and three females) over a one-week period was individually collected and analyzed for the presence of endogenous GHB and creatinine. The results of the non-normalized and normalized concentrations were statistically analyzed. Non-normalized GHB concentrations ranged from 0.00 to 6.63 pg/mL over seven days. The coefficients of variation (CV) for the individual non-normalized data were 44.0% to 77.7%. When the data were normalized to creatinine, the concentrations ranged from 0.00 to 6.79 pg/mg. The CVs for the creatinine-normalized results were between 29.7% and 76.8%. Analysis of the differences in CVs by the paired t-test (α = 0.05) found these improvements to be statistically insignificant. Such normalization allows for correction of urinary dilution or concentration by the kidneys which may affect endogenous GHB concentrations. The data also suggest significant (p < 0.001) differences in median endogenous urinary concentrations of GHB between males and females using the Mann-Whitney test. Because of the small number of subjects in this study, further investigations are required to substantiate this observation. Some of the subjects in this study demonstrated a strong tendency to produce higher or lower GHB concentrations at consistent periods during the day. This was most evident when looking at the creatinine-normalized concentrations. The results of our study indicate that there are significant intra- and interindividual variations in the urinary concentrations of endogenous GHB. Furthermore, there are also wide variations between individuals in the total daily amount of GHB excreted in the urine. Nonetheless, no specimen's GHB concentration approached 10 pg/mL (non-normalized) or 10 pg/mg (normalized). This study of the variability in endogenous urinary GHB excretion supports the recommendation of 10 pg/mL as an appropriate cutoff to identify exogenous GHB exposure in the absence of rare genetic deficiencies such as GHB aciduria. Patients with such a deficiency should be readily identifiable through prominent symptoms, repeated urinalysis, or genetic testing.

Introduction

Gamma-hydroxybutyrate (GHB), a naturally occurring metabolite of gamma-aminobutyric acid (GABA), has been studied for more than 40 years (1). In the 1970s, it was used for the treatment of sleep disorders and was reported to increase the release of growth hormone (2). This made it a popular steroid alternative with bodybuilders. More recently, GHB has become one of the many popular "club drugs." Currently, GHB has become one of the many popular "club drugs." Currently, GHB is legitimately available in the U.S. for the investigational treatment of narcolepsy and alcohol or opiate withdrawal (3). Because of the popularity of GHB as a recreational drug, it became a federally controlled Schedule I substance in the United States in March of 2000 (4). With its recent approval for clinical use, prescription GHB is a Schedule III substance.

Published reports following GHB abuse have shown the high concentrations that may be achieved in urine samples. For example, in one study, a driver found asleep in his car had a urinary GHB concentration of 1975 μg/mL approximately 2 h post-ingestion (5). Another study reported urine levels of 1085...
victims at concentrations that ranged from 2.3 \mu g/mL to 6100 \mu g/mL, respectively, in two impaired drivers (6). A GHB concentration of 141,000 \mu g/mL was measured in a comatose emergency room patient after ingestion of alcohol and GHB (7). These very high GHB concentrations rapidly decrease to near endogenous levels because of metabolism and renal excretion.

In the body, GHB is efficiently converted to succinic acid by oxidative enzymes and then metabolized through the Krebs cycle via succinic semialdehyde dehydrogenase (8). The plasma half-life has been reported to be an hour or less (9), thus, after ingestion of GHB, biological specimens for toxicological testing must be collected quickly to avoid confusion between exogenous and endogenous concentrations. Following a 1- to 5-g dose of GHB, concentrations fall to near endogenous levels 8 h or less in blood and 12 h or less in urine (3,10,11). In addition to GHB, abuse of gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) is on the rise, because both are rapidly biotransformed to GHB (12,13).

GHB aciduria is a rare genetic disorder resulting in a deficiency of succinic semialdehyde dehydrogenase producing an excessive accumulation of GHB. Endogenous GHB in patients with GHB aciduria have been reported to reach concentrations as high as 105 \mu g/mL in serum and 260 \mu g/mL in urine samples (14). Symptoms of patients with this disorder include mild to moderate mental retardation, ataxia, convulsions, optic atrophy, and speech disorders (15).

A recent report of the presence of GBL in wine may seem to confuse interpretations of endogenous GHB concentrations in biological fluids (16). However, the amount estimated in the wines are at such low amounts (5 \mu g/mL) that there should be no quantitative effect on the person consuming a typical amount of the product, nor should there be significant effects on endogenous GHB concentrations.

GHB has a strong CNS depressant effect because of its interaction with the GABA\(_A\) receptor and a GHB-specific receptor (17). Additionally, GHB may produce a number of other symptoms including confusion, dizziness, nausea, vomiting, amnesia, bradycardia, respiratory depression, and death (18).

Drug-facilitated sexual assaults (DFSA) occur after a victim is rendered unconscious or otherwise incapable of consenting to a sexual act through the voluntary or involuntary use of drugs (19). As a result of its strong sedative and amnesic effects, GHB has been implicated in a number of DFSA cases (19–26). The natural presence of GHB in the human body and its rapid elimination after ingestion can make it difficult to evaluate its role in cases of suspected GHB-facilitated assault; particularly when low concentrations are measured. For example, in one report GHB was identified in urine samples from alleged DFSA victims at concentrations that ranged from 2.3 \mu g/mL to 6100 \mu g/mL (26). The median concentration in 21 “positive” samples was only 15 \mu g/mL.

It has been suggested that urine is the most valuable specimen in DFSA cases (9,11,19). Given the reporting delay that surrounds these crimes and the extended detection window afforded by urine, these specimens are particularly important when GHB, GBL, or 1,4-BD is the suspected drugging agent. However, low levels of GHB in urine specimens (e.g., endogenous concentrations) may fluctuate considerably as the urine becomes diluted (i.e., from excessive drinking) or concentrated (i.e., from dehydration).

This study was designed to determine urinary concentrations of endogenous GHB over a one-week period, the variations that occur within those concentrations, and whether those variations are affected by normalization to urinary creatinine. Its purpose was to ascertain whether the fluctuation of endogenous concentrations was such that an endogenous urinary cutoff of 10 \mu g/mL or (10 \mu g/mg, if creatinine-normalized) may be proposed to avoid misrepresentation as being due to GHB ingestion.

### Materials and Methods

#### Materials

Na-GHB was acquired from Sigma Chemical (St. Louis, MO) and its purity was verified to exceed 99.5% by gas chromatography–mass spectrometry (GC–MS). Methanolic GHB-d\(_6\) was purchased from Radian International LLC (Austin, TX) as a 1-mg/mL solution of the sodium salt. The solution was diluted with HPLC-grade methanol (Fisher, Fair Lawn, NJ) to yield a working concentration of 100 \mu g/mL. Methylene chloride (Mallinkrodt, Paris, KY) was nanograde and concentrated sulfuric acid (J.T. Baker, Phillipsburg, NJ) was ACS-reagent grade.

#### Standards and controls

After correction for the sodium salt, aqueous GHB calibrators were prepared at concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 \mu g/mL. Negative controls consisted of deionized water. Positive controls were prepared in deionized water at concentrations of 1.0 and 10.0 \mu g/mL.

#### Experimental design

The National Institute on Drug Abuse (NIDA) Institutional Review Board approved the experimental protocol. All subjects provided informed consent, were paid for their participation, and resided on the closed research ward under continuous medical surveillance. Every urine void produced over a one-week period by eight GHB-free subjects (five males and three females) was individually collected and analyzed for the presence of endogenous GHB. Specimens were frozen at −20°C or colder until analysis to minimize any in vitro formation of GHB from putrefactive products (27). No preservatives were added to the specimens. The maximum storage time before analysis was nine months (Subject 8) with all other analyses occurring within six months of collection.

#### Extraction procedure

Quantitative analysis was carried out through the use of a previously published headspace GC–MS with electron impact ionization (GC–MS[EI]) analytical procedure that was optimized for enhanced sensitivity and precision at low concentrations (27,28). Briefly, 5-mL aliquots of each specimen, calibrator, and control were spiked with 0.1 mL of the 100-\mu g/mL solution of GHB-d\(_6\) and treated with 150 \mu L of concentrated H\(_2\)SO\(_4\). After a 5-min incubation period, the samples were extracted with 5 mL of methylene chloride for 10 min.
Following centrifugation for 5 min at 2500 rpm, the upper (aqueous) layer was removed to waste. The methylene chloride layer was transferred to conical-bottom test tubes and concentrated to ~75 μL under nitrogen at 35°C. This remaining methylene chloride was transferred to a 20-mL headspace vial for analysis by headspace GC–MS(EI) in full-scan mode.

**GC–MS(EI) analysis**

The GC–MS(EI) analyses were performed on a Hewlett-Packard (HP) 7694 headspace autosampler, an HP 6890 GC, and HP 5973 MS operated in the full-scan electron-impact mode (m/z 35–200). The samples were heated in the autosampler for 15 min at 100°C prior to injection. A J&W Scientific (Folsom, CA) DB-624 capillary column (30 m x 0.25 mm x 1.4 μm) was used with helium as the carrier gas (linear velocity = 34 cm/s). A solvent delay of 5.8 min allowed for elution of the methylene chloride. The electron multiplier was operated at 200 eV above the autotune setting. Retention times for the converted GHB and its deuterated analogue were approximately 10.9 min.

The analytical procedure has been shown to have an accuracy...
of 95% or better and imprecision of 7.9% (coefficient of variation) or less (27). The LOD for the procedure was 0.06 μg/mL and the LLOQ was 0.19 μg/mL (27). Evidence of carryover became apparent at concentrations near 10 μg/mL, although the procedure demonstrated good linearity (R² > 0.99) between 0.2 and 20 μg/mL. Further, beta-hydroxybutyrate, an elevated ketone body in diabetics, was found to not interfere with this assay.

Quantitative analysis of GHB

Quantitation was performed by construction of six-point calibration curves from 0.20 to 10.0 μg/mL. The calibration curves were ratios of the integrated area of the molecular ion of GBL (m/z 86) to the integrated area of the molecular ion of GBL-d₆ (m/z 92) as a function of analyte concentration. The resulting data were fit to a linear least-squares regression curve. Results of the patient specimens were compared to the calibration curve for determination of the individual endogenous concentrations of GHB within each specimen. A negative or positive quantitative (1.0 or 10.0 μg/mL) control was analyzed as every 10th sample within a batch run. These concentrations for the positive controls demonstrated acceptable quantitative results at both

### Table I. Summary of Non-Normalized Urinary Concentrations of Endogenous GHB in Eight Subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Race*</th>
<th>Age</th>
<th>n</th>
<th>Mean ± Std Dev</th>
<th>CV</th>
<th>Median</th>
<th>Range</th>
<th>Mean Daily Excreted GHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>AA</td>
<td>41</td>
<td>42</td>
<td>0.63 ± 0.34 μg/mL</td>
<td>53.9%</td>
<td>0.57 μg/mL</td>
<td>0.19-1.65 μg/mL</td>
<td>932 ± 148 pg</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>AA</td>
<td>44</td>
<td>52</td>
<td>3.02 ± 1.52 μg/mL</td>
<td>50.3%</td>
<td>2.59 μg/mL</td>
<td>0.63-6.63 μg/mL</td>
<td>4807 ± 1603 pg</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>AA</td>
<td>22</td>
<td>35</td>
<td>0.56 ± 0.31 μg/mL</td>
<td>56.0%</td>
<td>0.53 μg/mL</td>
<td>&lt; 0.19-1.94 μg/mL</td>
<td>425 ± 106 pg</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>C</td>
<td>24</td>
<td>35</td>
<td>0.38 ± 0.30 μg/mL</td>
<td>77.7%</td>
<td>0.27 μg/mL</td>
<td>0.00-1.28 μg/mL</td>
<td>1131 ± 673 pg</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>AA</td>
<td>40</td>
<td>52</td>
<td>2.48 ± 1.09 μg/mL</td>
<td>44.0%</td>
<td>2.22 μg/mL</td>
<td>1.05-6.01 μg/mL</td>
<td>2803 ± 1276 pg</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>AA</td>
<td>40</td>
<td>81</td>
<td>0.19 ± 0.14 μg/mL</td>
<td>75.7%</td>
<td>0.12 μg/mL</td>
<td>0.00-0.93 μg/mL</td>
<td>439 ± 192 pg</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>AA</td>
<td>34</td>
<td>62</td>
<td>0.52 ± 0.30 μg/mL</td>
<td>58.7%</td>
<td>0.52 μg/mL</td>
<td>0.00-1.70 μg/mL</td>
<td>581 ± 254 pg</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>AA</td>
<td>37</td>
<td>66</td>
<td>0.28 ± 0.19 μg/mL</td>
<td>66.6%</td>
<td>0.25 μg/mL</td>
<td>0.00-0.96 μg/mL</td>
<td>480 ± 131 pg</td>
</tr>
</tbody>
</table>

**Males:** 1.59 ± 1.42 μg/mL, 89.7% 1.07 μg/mL, 0.00-6.63 μg/mL, 1962 ± 1826 pg

**Females:** 0.31 ± 0.23 μg/mL, 80.9% 0.23 μg/mL, 0.00-1.70 μg/mL, 499 ± 205 pg

**All Data:** 1.01 ± 1.24 μg/mL, 122.3% 0.51 μg/mL, 0.00-6.63 μg/mL, 1361 ± 1576 pg

* Abbreviations: AA, African-American and C, caucasian.

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### Figure 2. Time period of daily high and low concentrations of endogenous GHB (see text).
the concentration in which most endogenous GHB concentrations were measured (near 1.0 µg/mL) and at the proposed cutoff concentration between endogenous and exogenous GHB (10.0 µg/mL). Positive controls were considered acceptable if they were within ±20% of their targeted value. Solvent blanks were placed after all 10.0 pg/mL positive controls as a precaution to prevent carryover into patient samples.

**Determination of creatinine concentrations**

The urine creatinine concentration was determined using a Beckman Synchon CX7 Delta System. The analyzer was properly calibrated prior to use and appropriate controls were run to evaluate its performance. Imprecision for the creatinine assay on this instrument was 10% at 0.5 mg/dL and 2.5% at 7.0 mg/dL.

**Results and Discussion**

Figures 1A–H show the GHB concentration versus time data for the eight subjects. Table I summarizes the demographic and non-normalized analytical data for the eight subjects. Subject 2 maintained the highest urinary concentrations of endogenous GHB with a mean concentration of 3.02 µg/mL. Subject 6 had the lowest concentration of endogenous GHB, with a number of specimens containing no detectable GHB or an amount below the LLOQ. Non-normalized interindividual data analyzed by the Kruskal-Wallis one-way analysis of variance by ranks indicated that median GHB concentrations between the individual subjects were significantly different from one another (p < 0.001). This test does not assume normal distribution of the data.

The males in this study ranged from 0.00 to 6.63 µg/mL (X = 1.59 µg/mL) and the females ranged from 0.00 to 1.70 µg/mL (X = 0.31 µg/mL). The difference in medians between genders was determined to be significant (p < 0.001) using the Mann-Whitney test. This is a nonparametric test that assumes the data are independent random samples from two populations that have essentially the same variance. Although the variance between males and females appears to differ quite substantially, this test was chosen over the two-sample t-test because the endogenous GHB concentrations do not appear to be normally distributed. This difference in medians is an interesting observation, as most victims of DFSA are female. However, with only three female subjects in this study, further investigation into these observed differences between males and females are required to substantiate this observation.

Table I also presents the average daily total amount of GHB excreted in the urine for each subject. This was determined by multiplying the sample volume produced by the measured GHB concentration within that sample. The daily totals were calculated for each subject and the daily averages were determined from these totals. As can be seen in the table, the total amount of daily GHB varied widely from one individual to the next.

The data were further evaluated to determine the effect of creatinine normalization of GHB concentrations on the variability of endogenous GHB concentrations. Figures 1A–H and Table II show the results of such normalization. For the most part, the data compare well to that of the non-normalized data. The CVs for all of the data are similar in the non-normalized (122.3%) and the creatinine-normalized concentrations (121.7%). The individual CVs for the creatinine-normalized data ranged from 29.7% (Subject 5) to 76.8% (Subject 8). The paired t-test was used to analyze the differences in the individual CVs of measured concentrations of endogenous GHB before and after creatinine-normalization. This test was used assuming normal distribution of the differences between the CVs. The results suggest that the differences are statistically insignificant (α = 0.05); thus, there is no statistical advantage or disadvantage in normalizing endogenous GHB concentrations to urinary creatinine. However, this does not suggest that creatinine-normalization is not beneficial. Such an endeavor removes the effect that urine dilution has on the interpretation of endogenous concentrations. This may become an issue when the subject is drinking heavily or is dehydrated.

There is no published indication of a hormonal influence on the release of endogenous GHB. Nonetheless, the data from this study were examined for evidence of a diurnal effect on urinary concentrations of endogenous GHB. This was accomplished by categorizing the two highest and two lowest daily concentrations of endogenous GHB (both normalized and non-normalized) from each subject into 4-h intervals. The percent of high and low concentrations falling within a particular time range was graphed to discern trends in the data (Figure 2).

The non-normalized lowest levels were evenly distributed throughout the day (Figure 2A). When the data were normalized (Figure 2B), nearly 30% of the lowest endogenous concentrations occurred during the 8:00 p.m.
to midnight time period. Over one-third of the highest concentrations occurred during the 12:00 noon to 4:00 p.m. time period (Figures 2C–D).

Although the trends observed when looking at the data from all of the subjects do not provide proof of a diurnal pattern for endogenous levels of GHB, the data from some of the individuals demonstrate a strong tendency to produce high and low concentrations at consistent periods during the day. This was most evident when looking at the creatinine-normalized concentrations. For example, Subject 1 (Figure 1A) always produced urine samples with the highest daily concentrations of GHB during the 8:00 a.m. to 8:00 p.m. time period. Of those specimens with the highest concentrations, 57% were provided between 12:00 noon and 4:00 p.m. Conversely, during the one-week period of this study, the lowest normalized endogenous concentrations occurred during the 12:00 noon to 4:00 p.m. time period.

Subject 6 (Figure 1F) also demonstrated a tendency to provide specimens with the highest daily normalized GHB concentrations between 12:00 noon and 4:00 p.m. (69%). Further, this subject provided 81% of the specimens with the lowest endogenous concentrations during the 8:00 p.m. to 8:00 a.m. time period.

Subject 8 (Figure 1H) provided 76% of the specimens with the highest daily endogenous GHB levels between 8:00 a.m. and 8:00 p.m. and 100% of the specimens with the lowest concentrations between 8:00 p.m. and 8:00 a.m.

In cases of DFSA, when the victim’s urine tests “high” for GHB, it has become a practice for some communities to collect a second urine specimen from the victim (at some later time) to serve as a “control”. The results of this study show that an individual’s endogenous level of urinary GHB can vary quite significantly over the period of one week. Therefore, such practices should be used with caution to avoid misinterpretation of GHB findings.

Two potential limitations require consideration before drawing conclusions from these data. The first limitation is that there were only three females that participated, making it difficult to fully evaluate the observed differences in endogenous GHB concentration between sexes. Further, seven of the eight subjects in the study were African Americans. If ethnic variations exist in endogenous GHB concentrations, the results of this study may be less representative of the general population. Additional studies on random urine collections from a varied population are ongoing and will help clarify this issue.

Finally, although wide ranges of endogenous GHB concentrations in the urine of the subjects were observed, no specimen’s GHB concentration approached 10 µg/mL (non-normalized) or 10 µg/mg (normalized). As a result of this study, the authors recommend that a minimum GHB concentration of 10 µg/mL be used as an appropriate differentiation between endogenous and exogenous GHB.

References

23. Gamma-hydroxybutyrate, flunitrazepam and ketamine. Drug Enforcement Administration Testimony Before the House Commerce
Subcommittee on Oversight and Investigations, 106th Congress (March 11, 1999) (Woodworth T).


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