Endogenous production complicates the interpretation when gamma-hydroxybutyric acid (GHB) is measured in urine for forensic purposes. We performed a cross-sectional study to test the hypothesis that pregnant women have higher levels of urinary GHB than non-pregnant controls, and thus increased risk of false-positive GHB tests. GHB, gamma-butyrolactone (GBL) and beta-hydroxybutyric acid (BHB) concentrations in urine from 66 pregnant women and 69 non-pregnant controls were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS–MS). The mean GHB, GBL, and BHB concentrations were 0.36, 0.34 and 1.92 mg/L in the pregnant women, and 0.24, 0.08 and 0.40 mg/L in the control group. The pregnant women had significantly higher levels of GHB (1.5-fold), GBL (4.3-fold), and BHB (4.8-fold). Creatinine-adjusted GHB concentrations were similar in both groups. Pregnant women have higher urinary levels of GHB, GBL, and BHB. In LC–MS–MS assays not distinguishing between GHB and BHB, there is a significantly increased risk of false-positive GHB tests in pregnant women. This false-positive rate can be reduced by correcting for creatinine concentration, by using GHB-specific assays or by introducing higher interpretative cut-off levels for pregnant women in assays that do not discriminate between GHB and BHB.

Introduction

Gamma-hydroxybutyric acid (GHB) occurs naturally in all mammals, but its function remains unknown. Being both a precursor and a metabolite of gamma-aminobutyric acid (GABA) (1), it is anticipated that GHB acts as a central nervous system (CNS) neuromodulator, mediating its effects through GABAB and GHB-specific receptors, or by affecting dopamine transmission (2).

GHB has been used recreationally since it was first marketed in 1960. It is labeled as an illegal drug in most countries, but also used as a legal drug (Xyrem) in patients with narcolepsy (1–4). In high doses, GHB inhibits the CNS, inducing sleep and inhibiting the respiratory drive (2). In lower doses, its euphoriant effect predominates (5). The toxicity of GHB is generally considered to be low, but several GHB-related fatalities have been reported (6–9). Pregnant women with a history of drug abuse are often subjected to urine analysis to reveal intake of substances that could damage the fetus. The presence of endogenous GHB in urine makes an interpretative cut-off necessary to differentiate physiological GHB concentrations from concentrations seen with exogenous ingestion. Although 10 mg/L is the most commonly proposed cut-off (2,10–14), levels ranging from 2 to 20 mg/L have been suggested (3). Few studies have taken urine dilution into consideration, but in one study on 50 females, 323 µg GHB/mmol creatinine was indicated as an upper limit for normal GHB concentration, and 1000 µg GHB/mmol creatinine was proposed as an interpretative cut-off (3). In another study, no statistical difference was found between non-normalized and creatinine-normalized urine GHB results in samples collected during one week from eight persons (10).

When measuring GHB in urine, several complicating factors should be addressed. Firstly, endogenous gamma-butyrolactone (GBL) is converted into GHB, and vice versa, in a pH-dependent process, and several GHB assays involve a step converting all GHB into GBL before analysis (2,10,11,15). Secondly, there is a naturally occurring ketone body, beta-hydroxybutyric acid (BHB), which is an isomer of GHB (14). Liquid chromatography–tandem mass spectrometry (LC–MS–MS) is increasingly being applied for GHB analysis. BHB and GHB have identical molecular weights (MW) and fragment into identical daughter ions under LC–MS–MS conditions, making them indistinguishable in an MS. If this is not taken into account, by separating the isomers chromatographically using a column that can retain small polar organic molecules, for example, the measured GHB concentrations are, in reality, BHB and GHB combined. We have reason to believe that such methods have been applied even for forensic purposes. There also exists an alpha-isomer, alpha-hydroxybutyric acid (AHB) of GHB, which is elevated in diabetics (14). Thirdly, there is firm evidence that GHB is produced in vitro, and unless the urine samples are stored at low temperatures shortly after voiding.
the GHB concentration might show a large increase (15–17). 1,4-Butanediol (1,4-BD) is a chemical precursor of GHB that sometimes is detected in GHB abusers (2,5). One study detected 1,4-BD in postmortem human brain tissue, indicating that 1,4-BD might be endogenously produced (18).

We have experienced that urine samples from pregnant women tend to have higher GHB concentrations than samples from other persons with no assumed GHB intake. If this is true, pregnant women will have a higher risk of false-positive tests. It is important to identify this, as it may affect treatment and could lead to serious legal consequences. To our knowledge, urine GHB concentrations in pregnant and non-pregnant have not previously been systematically compared.

Our main objective was to test the hypothesis that pregnant women have higher urinary concentrations of GHB, GBL, and/or BHB than non-pregnant controls (male and female). Furthermore, we wanted to examine the effect of duration of pregnancy and use of hormonal contraceptives on the endogenous levels of these compounds.

Materials and Methods

We performed a cross-sectional study comparing the GHB, GBL, and BHB concentrations in pregnant women and non-pregnant controls. The pregnant women were recruited from the midwife center of Tromsø municipality in northern Norway, from October 2007 to March 2008. The non-pregnant controls were nursing, dentist, and medical students at Tromsø University College and University of Tromsø, recruited from August through November 2007. The study was approved by the local ethics committee.

Midwives were instructed to ask pregnant women in their second or third trimester attending routine appointments whether they wanted to participate in the study. The women were given questionnaires, consent forms, and urine sampling tubes that they were to bring along at the next appointment if they agreed to participate. The non-pregnant controls were recruited by informing students about the study in connection with a lecture. The urine samples, questionnaires, and consent forms from the volunteers were collected shortly after the lecture. Both males and non-pregnant females were included because the cut-off values for analysis of drugs of abuse generally are not gender-specific. As females were in the majority among all included students, substantial power for subgroup analysis of female controls was expected. Urine samples were registered and frozen at ~80°C as soon as possible after voiding. All samples were screened for amphetamines, benzodiazepines, cannabinoids, cocaine, and opiates by immunoassay (CEDIA techniques, Microgenics, Fremont, CA). Participants with samples containing any of these drugs were excluded. The urine samples from female controls were tested for human chorionic gonadotropin (hCG) (ClearView hCG, Inverness Medical, Bedford, U.K.) to exclude any unknown pregnancies. Written consent was required from all participants.

The main outcomes were GHB, GBL, and BHB concentrations in urine, both unadjusted and creatinine adjusted. There is considerable intranidividual variation in diuresis and thus in the concentration of renally excreted substances. This can be accounted for by normalizing the measured concentrations to creatinine concentrations, assuming a constant rate of creatinine excretion (19). The combined concentrations of GHB+GBL and GHB+BHB were included as outcome variables to make the results comparable to studies not differentiating GHB from GBL or BHB. The concentration of GHB+GBL (in mg/L) was calculated from the sum of molar concentrations of GHB and GBL using the MW of GHB. The MWs of the isomers GHB and BHB are identical, whereas GBL has a lower MW. 1,4-BD was also analyzed, but not included as an endpoint because we expected to find it in negligible concentrations.

Secondary outcomes included GHB, GBL, and BHB levels in pregnant women compared to female and male controls separately. Correlations between GHB concentrations and duration of pregnancy and use of hormonal contraceptives were also examined. Post hoc, we decided to examine the false-positive rate in pregnancy and non-pregnancy at different cut-off levels.

Analytical methods and chemicals

Analytical-grade formic acid and HPLC-grade methanol were supplied by Merck (Darmstadt, Germany). GHB sodium salt and GHB-d6 in methanol (1 mg/mL) were from Lipomed (Arlesheim, Switzerland). GBL-d5 (1 mg/mL in acetonitrile) was purchased from Cerilliant (Round Rock, TX) and 1,4-BD was from Riedel-de Haën (Seetze, Germany). GBL was from Fluka (Steinheim, Germany) and BHB was from Aldrich (Steinheim, Germany). Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA). A mixed stock solution with GHB, GBL, BHB, and 1,4-BD was prepared by adding the compounds to synthetic urine to a final concentration of 104 mg/L. Synthetic urine was prepared according to the method of Kark et al. (20) with some modifications. Standards were prepared by dilution of the stock solution with synthetic urine at the following concentrations: 6.66, 3.33, 1.66, 0.83, 0.42, 0.21, and 0.105 mg/L. Quality control (QC) samples at 5.2 and 0.52 were prepared in the same manner. The samples, standard solutions, and QCs were stored at ~80°C before analysis. Internal standard solution was prepared by adding GBL-d5 and GHB-d5 to Milli-Q water to a final concentration of 8 mg/L.

Analyses of GHB, BHB, 1,4-BD, and GBL were performed in a similar manner as previously described by Wood et al. (21) on a Waters Acquity UPLC (Waters, Milford, MA) interfaced to Waters Micromass Quattro Premier XE tandem-quadrupole MS (Waters, Manchester, U.K.). The system was controlled by MassLynx™ version 4.1. The chromatography was performed on a 2.1- × 100-mm Waters Acuity HSS T3 (C18) column maintained at 50°C. The mobile phase consisted of 8% methanol in 0.5 g/L aqueous formic acid with a flow rate of 0.4 mL/min (isocratic). The method was validated and showed good linearity from 0.1 to 83 mg/L ($r^2 > 0.997$) for GHB, and from 0.1 to 30 mg/L ($r^2 > 0.992$) for BHB, GBL, and 1,4-BD. Limits of detection (LOD) and quantification (LOQ) for GHB and 1,4-BD were found to be 0.04 and 0.07 mg/L, respectively. For GBL and BHB, the LOD and LOQ were 0.06 and 0.1 mg/L, respectively. Accuracy was 101.1% with a relative standard de-
viation (RSD) of 6.6% at 5–33 mg/L, and intermediary precision varied from 3.2 to 5.6% RSD for GHB. The creatinine concentrations were determined by using a method based on the Jaffe reaction (Microgenics DRI Creatinine-Detect Test, Microgenics and Olympus AU400) (22).

Possible confounders
Potential confounders that were recorded include age, body mass index (BMI), smoking status, time since last alcohol intake, use of hormonal contraceptives by female controls, and number of days to term predicted by ultrasound in the pregnant women.

Several studies have examined determinants for physiological GHB concentrations (2,3,4,12,14). Considerable intraindividual and interindividual variations in urine GHB concentrations have been reported (4,10,12). Some authors reported gender differences (10), whereas others did not find such differences (4), but these studies were limited by low sample sizes. In a large study, no difference in median GHB concentration was found between males (n = 130) and females (n = 77) (2). There may also be differences in endogenous GHB concentration related to ethnicity (4,10), but another study (n = 207) did not report such differences (2). In one study (n = 50), urinary GHB concentrations decreased with age in women, but this age effect was compensated for when correcting for creatinine concentrations (3). Another study found no significant age differences (2). Factors such as diet or diabetes mellitus do not seem to influence GHB in urine (2,12,14). Another small study (n = 15) found no significant effect of smoking or drinking alcohol on GHB concentrations (4).

We expected the non-pregnant controls to be younger and have a lower BMI than the pregnant women. Smoking, alcohol consumption, and the use of therapeutic drugs and oral contraceptives are potential confounding factors that are likely to be less frequent in the pregnant population. Age and height are the only identified possible confounders that are unrelated to pregnancy.

Study size
In 50 previous samples analyzed at our laboratory, the average creatinine-adjusted GHB concentration was 170 µg GHB/mmol creatinine. The standard deviation was 152 µg GHB/mmol creatinine. To detect a difference of 85 µg GHB/mmol creatinine (50%), with a significance level of 0.05 and a power of 0.9, we needed 67 participants in each group. These samples were analyzed with an assay not differentiating GHB from BHB, and the concentrations used in the study size calculation were higher than what we expected from the more refined assay used in the cross-sectional study. We aimed to include at least 50 female controls, achieving a power of 0.8 for subgroup analysis of female controls. The false-positive rate for GHB was examined at four different previously proposed cut-off levels.

Table I. Descriptive Data

<table>
<thead>
<tr>
<th></th>
<th>Pregnant Women (n = 66)</th>
<th>Controls (n = 69)</th>
<th>Female Controls (n = 52)</th>
<th>Male Controls (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>30.7</td>
<td>24.7*</td>
<td>24.7*</td>
<td>24.9*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.2</td>
<td>68.3*</td>
<td>64.0*</td>
<td>81.3*</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>166</td>
<td>170*</td>
<td>167</td>
<td>183*†</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>27.6</td>
<td>23.3*</td>
<td>23.0*</td>
<td>24.2*</td>
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<tr>
<td>Urine creatinine concentration (mmol/L)</td>
<td>9.84</td>
<td>7.86*</td>
<td>6.86*</td>
<td>10.92†</td>
</tr>
<tr>
<td>Urine pH</td>
<td>6.33</td>
<td>6.62*</td>
<td>6.67*</td>
<td>6.48</td>
</tr>
</tbody>
</table>

* p < 0.05 compared to pregnant women (independent sample t-test).
† p < 0.05 compared to female controls (independent sample t-test).
‡ BMI: body mass index.

Table II. Concentrations of GHB, GBL, BHB, GHB+GBL, and GHB+BHB (mg/L) in Pregnant Women and Controls

<table>
<thead>
<tr>
<th></th>
<th>Pregnant Women (n = 66)</th>
<th>Controls (n = 69)</th>
<th>Difference for Mean (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHB</td>
<td>Mean 0.36</td>
<td>0.24</td>
<td>0.12 (0.04–0.19) p = 0.002</td>
</tr>
<tr>
<td></td>
<td>Median 0.32</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range 0–1.06</td>
<td>0–1.27</td>
<td></td>
</tr>
<tr>
<td>GBL</td>
<td>Mean 0.34</td>
<td>0.08</td>
<td>0.26 (0.14–0.39) p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Median 0.21</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range 0–2.32</td>
<td>0–1.79</td>
<td></td>
</tr>
<tr>
<td>BHB</td>
<td>Mean 1.92</td>
<td>0.40</td>
<td>1.53 (0.85–2.20) p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Median 0.90</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range 0–14.07</td>
<td>0–8.79</td>
<td></td>
</tr>
<tr>
<td>GHB+GBL</td>
<td>Mean 0.70</td>
<td>0.32</td>
<td>0.38 (0.22–0.54) p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Median 0.54</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range 0–3.14</td>
<td>0–2.24</td>
<td></td>
</tr>
<tr>
<td>GHB+BHB</td>
<td>Mean 2.28</td>
<td>0.71</td>
<td>1.64 (0.94–2.34) p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Median 1.43</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range 0–14.89</td>
<td>0–9.24</td>
<td></td>
</tr>
</tbody>
</table>
Statistical methods

Independent sample t-tests were used to compare the difference of means in unadjusted and creatinine-adjusted GHB, GBL, and BHB concentrations with a 95% confidence interval (CI) between pregnant women and all controls and by controls stratified by gender. The difference of means with a 95% CI was also used to compare unadjusted and creatinine-adjusted GHB, GBL, and BHB concentrations between female users and non-users of hormonal contraceptives in the control group. Simple linear regression was used to examine correlations of adjusted or unadjusted GHB concentrations to duration of pregnancy. The covariates age, height, and urinary pH were controlled by multivariable linear regression. We used odds ratios (OR) to examine the false-positive rate in pregnancy and non-pregnancy at different cut-off levels. Statistical tests were performed using Statistical Package for the Social Sciences (SPSS) version 14.0.

Results

Of the 68 pregnant women who wanted to participate in the study, 66 were found eligible for analysis. One was excluded because of a missing questionnaire and one because of a missing consent form. Of the 72 non-pregnant controls who wanted to participate, 69 were found eligible for analysis. Two were excluded because of positive pregnancy tests and one because of a positive benzodiazepine test.

Descriptive data of the study participants are given in Table I. The pregnant women had a mean age of 30.6 years compared to 24.7 years in the control group. Other recognized baseline differences in weight, height, smoking and drinking habits, and use of hormonal contraceptives could be attributed to pregnancy status or gender.

Main results

GHB, GBL, and BHB urine concentrations in pregnant women and all controls are given in Table II, and creatinine-adjusted GHB, GBL, and BHB concentrations are presented in Table III. All unadjusted concentrations were significantly higher in the pregnant women than in the control group. When adjusting for creatinine, there was no difference in GHB concentrations, but GBL, BHB, GHB+GBL, and GHB+BHB were significantly higher in the pregnant women. When stratifying the non-pregnant group by gender (Figures 1 and 2), the female, but not the male group had significantly lower mean unadjusted GHB concentrations than the pregnant women. There was no difference in creatinine-adjusted GHB concentrations between pregnant women or male or female controls. Both male and female controls had significantly lower crude and creatinine-adjusted GBL, BHB and GHB+BHB concentrations than the pregnant women. GHB+GBL corrected for creatinine was significantly lower in male but not female controls compared to the pregnant women. One sample, from a female control, had extremely high creatinine-adjusted concentrations of all substances. Creatinine-adjusted GHB in this person was more than two times higher than the highest observed in the pregnant population. Post
hoc analysis excluding this person did not significantly change our main findings. 1,4-BD was not detected in any of the samples.

Other analysis

Multivariable linear regression revealed no association between unadjusted or creatinine-adjusted GHB, GBL or BHB concentration and age, height, or urine pH (data not shown). All pregnant women were in their second or third trimester, and the mean length of pregnancy was 221.1 days (range 162–277 days). The duration of pregnancy did not affect the GHB concentrations (data not shown). There was no difference in unadjusted or creatinine-adjusted GHB, GBL or BHB concentrations between the genders (Figures 1 and 2) or between female users and non-users of hormonal contraceptives in the control group (data not shown).

All measured GHB and GHB+GBL concentrations were below the standard 10 mg/L interpretative cut-off level, but in two (3.0%) samples from the pregnant group, the GHB+BHB concentrations were above this limit. Table IV shows the false-positive rates for GHB+BHB concentrations at different previously suggested cut-offs (2,10–14). The odds ratios for false-positive GHB+BHB tests among pregnant women were significantly higher at low cut-off values. None of the samples had creatinine-adjusted GHB or GHB+GBL concentrations above the suggested cut-off at 1000 µg/mmol creatinine, but creatinine-adjusted GHB+BHB gave false-positive rates at 6.1% among the pregnant women and 1.4% in the control group (OR: 4.4, 95% CI: 0.5–40.3).

Discussion

Our study confirms the hypothesis that pregnant women have higher average urine levels of not only GHB (1.5-fold), but also GBL (4.3-fold) and BHB (4.8-fold). However, when adjusting for creatinine, the GHB concentration is similar in pregnant women and the control group. GBL and BHB remain elevated among the pregnant women even after correcting for creatinine.

Fortunately, most published GHB assays have no interference from BHB, and for these methods endogenous GHB or GHB+GBL levels in pregnancy are expected to be well below the recommended cut-off limit. However, our results show that LC–MS–MS assays that measure the sum of GHB and BHB have an increased risk of false-positive tests in pregnant women.

One important limitation of this study is the difficulty in defining a control group where pregnancy is the only different characteristic. Even in prospective studies with fertile women being their own controls before or after pregnancy, there will be bias due to pregnancy-induced behavioral changes. These give rise to potential confounding factors such as different smoking or drinking habits. Our control group consisted of students that were not matched to the pregnant women. There were indeed significant differences in age, the pregnant women being older, but according to earlier reports, this should not affect the creatinine-adjusted concentrations (3), and in one comprehensive study on variations of urinary endogenous GHB, there was no age effect in the age range of our participants (2).

The observed GHB concentrations in both the pregnant and the non-pregnant population were somewhat lower than what has been found by others (3,10,12–14). One possible explanation is that in vitro production of GHB was minimized by freezing the samples at –80°C shortly after voiding. In vitro production of GHB in urine is well known, and is dependent on time to analysis and storage temperature (15,16). Our assay measures GHB and GBL separately, whereas other methods involve a step in which GHB is converted to GBL before analysis. When using the sum of GHB and GBL, our range of concentrations in the non-pregnant group (0–2.24 mg/L) was similar to LeBeau et al. (2) (0–2.70 mg/L). Our results will also be lower than results from assays not differentiating between GHB and BHB. Finally, there could be unknown common environmental factors among our study participants that could influence the levels of GHB, GBL, and/or BHB.

The collection of urine samples from the pregnant women and non-pregnant controls was performed at different loca-
tions. Longer time from voiding until the urine sample was frozen among the pregnant women could be one explanation for the higher GHB concentration in these samples. The exact time between voiding and freezing was not recorded, but all collectors were instructed that the urine samples should be frozen as soon as possible after voiding.

To our knowledge, this is the first study designed to examine physiological GHB, GBL, and BHB concentrations in urine from pregnant women. One study (n = 207) reported that the GHB concentrations were within the normal range (0.12–0.72 mg/L, mean 0.36 mg/L, median 0.34 mg/L), but this study only included six women who were either pregnant or had recently given birth (2). Our results should encourage further studies to examine possible explanations for elevated concentrations of GHB, GBL, and BHB in pregnancy, such as increased production or increased renal excretion compared to other endogenous substances. Both maternal factors, such as latent diabetes mellitus in which BHB is shown to be elevated (14), or diabetes mellitus in which BHB is shown to be elevated (14), or increased renal excretion compared to other endogenous substances. Both maternal factors, such as latent diabetes mellitus in which BHB is shown to be elevated (14), or diabetes mellitus in which BHB is shown to be elevated (14), or renal disease, may lead to increased production and increased renal excretion of GHB. Our results imply that concentrations of endogenous GHB, GBL, and BHB in urine are indeed elevated in pregnant women in the second and third trimesters, although no significant differences were found for creatinine-adjusted GHB concentrations. The combined concentrations of GHB and BHB were above the 10 mg/L interpretative cut-off in two pregnant women. In order to minimize the risk of false-positive results when testing urine from pregnant women for legal purposes, we suggest that only methods specifically identifying GHB should be applied, a higher cut-off should be considered, and that creatinine-adjusted concentrations should be used. The robustness of our main results when controlling for potential confounding factors indicates that our findings also apply to pregnant women in their third and late second trimesters in general.

Acknowledgments

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