Renal Clearance of γ-Hydroxybutyric Acid in Rats: Increasing Renal Elimination as a Detoxification Strategy

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ABSTRACT

Intoxication with γ-hydroxybutyric acid (GHB) is associated with coma, seizure, and death; treatment of overdoses is symptomatic. The objectives of this investigation were to characterize the renal clearance and total clearance of GHB in rats and to evaluate potential strategies for increasing the elimination of GHB after drug overdoses. GHB was administered by i.v. infusion at low (108 mg/h/kg), medium (128 mg/h/kg), or high (208 mg/h/kg) doses. Crossover studies were performed under steady-state conditions using the medium dose in the absence or presence of L-lactate, pyruvate, D-mannitol, sodium bicarbonate, or normal saline. GHB in plasma and urine samples was assayed using liquid chromatography-tandem mass spectrometry. Infusion of the low, medium, and high doses of GHB produced steady-state plasma concentrations of 0.22 ± 0.04, 0.43 ± 0.05, and 0.68 ± 0.11 mg/ml. The renal clearance of the medium (51.8 ± 13.0 ml/h/kg) and high (97.1 ± 43.1 ml/h/kg) doses was significantly higher than that of the low dose (14.9 ± 5.1 ml/h/kg), whereas the total clearance values were significantly lower than that of the low dose. The renal clearance was significantly increased by the concomitant administration of L-lactate, pyruvate, D-mannitol, or sodium bicarbonate with GHB but was not altered by normal saline. The total and metabolic clearance values were significantly increased by all treatments except normal saline. Overall, our results indicated that the renal clearance of GHB is dose-dependent, involving capacity-limited reabsorption. Monocarboxylate transport inhibitors, osmotic diuresis using D-mannitol, or the administration of sodium bicarbonate can increase the renal and total clearances of GHB. The approaches used in this investigation may offer potential detoxification strategies for the treatment of GHB overdoses.

γ-Hydroxybutyric acid (GHB) (Fig. 1) is a naturally occurring short-chain fatty acid present in mammalian brain, formed primarily from the precursor, γ-aminobutyric acid (Roth, 1970). Endogenous GHB is also present in various mammalian tissues such as heart, liver, and kidney, where its function is unknown (Maitre, 1997). GHB was first synthesized in 1960 as a γ-aminobutyric acid analog capable of crossing the blood-brain barrier (Laborit, 1964). Two precursor compounds, γ-butyrolactone and 1,4-butanediol (Fig. 1), can be quickly converted to GHB via hydrolysis by various lactonases and oxidation by alcohol dehydrogenase in brain and peripheral tissues (Mason and Kerns, 2002). In the 1960s, GHB was initially used as an anesthetic drug, but a lack of analgesic effects destined the drug for failure. In the 1970s, GHB was found to be beneficial in the treatment of the sleep disorder narcolepsy (Mamelak et al., 1986), and in the 1980s, GHB was used to treat alcohol and heroin dependence in Europe (Gallimberti et al., 2000). In 1990, GHB was marketed as a dietary supplement in the United States, but it was rapidly banned by the Food and Drug Administration from over-the-counter sales in the same year due to widespread reports of its adverse effects (Galloway et al., 1997). At that time, bodybuilders used GHB as a popular steroid alternative because of its growth hormone-releasing effects (Okun et al., 2001). By the late 1990s, GHB was used as a recreational drug at nightclubs and rave parties for its euphoric effects (Wong et al., 2004). GHB has been also used as a means of drug-facilitated sexual assaults (date rape) due to its hypnotic/amnesic effects (Schwartz et al., 2000). In 2002, GHB (Xyrem) was approved by the Food and Drug administration as an orphan medication for treating a small population of patients with narcolepsy who experience episodes of cataplexy, but it was classified as a schedule III drug and its distribution was tightly restricted. The manifestations of GHB intoxication are primarily a result of its central nervous system and respiratory depres-
sion, and to a lesser extent, its effects on the cardiovascular and gastrointestinal systems. The adverse effects associated with GHB are dose-dependent and include nausea, vomiting, dizziness, bradycardia, hypotension, coma, seizure, and even death (Mason and Kerns, 2002). GHB or its prodrugs are especially toxic when mixed with alcohol and/or other recreational drugs to increase its euphoric effects. This combination has led to significant morbidity and mortality (Okun et al., 2001). However, the treatment of GHB overdose is very limited, consisting mainly of supportive care. There have been no specific antidotes reported for clinical use, although flumazenil, naloxone, and physostigmine have been tried (Mason and Kerns, 2002).

The pharmacokinetics of GHB has been found to be non-linear in rats (Lettieri and Fung, 1978a, 1979), mice (Pol et al., 1975), cats (Snead et al., 1976), dogs (Pol et al., 1975), and humans (Ferrara et al., 1992, 1996; Palatini et al., 1993; Scharf et al., 1998), with decreasing total clearance with increasing dose. The mechanisms underlying the nonlinear pharmacokinetics of GHB may include capacity-limited metabolism (Lettieri and Fung, 1976, 1978a, 1979; Ferrara et al., 1992; Palatini et al., 1993) and/or absorption of GHB (Arena and Fung, 1980). After the administration of low doses, the urinary recovery of GHB was determined to be 5 to 7% in rats (Lettieri and Fung, 1976) and <1% in humans (Ferrara et al., 1992). However, the renal clearance of GHB may be more important after overdoses, since it has been reported that intoxicated patients who ingested GHB, γ-butyrolactone, and 1,4-butanediol had high concentrations of GHB in urine samples (Zvosec et al., 2001; Sporer et al., 2003). Therefore, it is possible that the renal clearance of GHB may play a significant role in its elimination after overdoses.

The objectives of this study were to characterize the renal and total clearances of GHB in rats and to evaluate three treatment strategies to increase the renal clearance of GHB that may be useful in the treatment of GHB overdoses. These strategies are as follows. 1) Use of monocarboxylate transporter (MCT) substrates to inhibit the active renal reabsorption. GHB has been reported to inhibit pyruvate transport by both MCT1 and MCT2 (Lin et al., 1998). Two MCT substrates, pyruvate and lactate, are used in these studies to evaluate MCT-mediated inhibition of the renal reabsorption of GHB. 2) Osmotic diuresis to increase urinary flow and decrease passive reabsorption. Mannitol is administered to evaluate the effects of osmotic diuresis on GHB renal reabsorption. 3) Urine alkalinization to decrease the passive reabsorption of this acidic compound. Sodium bicarbonate is administered to produce alkalinization of the urine.

Materials and Methods

Chemicals and Reagents. GHB (as sodium salt), L-(-)-lactate (as sodium salt), pyruvate (as sodium salt), formic acid, anthrone, inulin, mannitol, and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). The internal standard GHB-D6 (Fig. 1) (as sodium salt dissolved in MeOH, 1 mg/ml) was purchased from Cerilliant (Round Rock, TX). Acetonitrile, methanol, acetic acid, and NaHCO3 were purchased from Fisher Scientific Co. (Fairlawn, NJ). The concentrated H2SO4 (93%) was purchased from J. T. Baker (Phillipsburg, NJ). Glacial acetic acid was purchased from EM Scientific (Gibbstown, NJ), 2,3-3[H]GHB was purchased from Moravek Biochemicals (Brea, CA).

Animals. Age-matched adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 260 to 350 g were used throughout the study. The rats were randomly assigned to groups and kept in individual cages. The animal housing room had controlled environmental conditions with temperature and relative humidity of approximately 20 ± 2°C and 40 to 70%, respectively, and artificial lighting, alternating on a 12-h light/dark cycle. All care and experiments were approved by the Institutional Animal Care and Use Committee at the University at Buffalo.

Animal Surgery and Sample Collection. The cannulae were inserted into rat right jugular veins, left femoral veins, and bladders after an i.m. injection of 90 mg/kg ketamine and 9 mg/kg xylazine (Henry Schein, Melville, NY). The animals were allowed 3 to 4 days for recovery from surgery. On the day of study, the rats were placed in metabolism cages for collections of urine. GHB, inulin, l-lactate, pyruvate, D-mannitol, or NaHCO3 dissolved in water, was administered via an i.v. bolus injection followed by an i.v. infusion at a rate of 20.6 µl/min through the femoral veins to rats (n = 4 each group). Blood samples (200 µl each) were drawn from the jugular vein at different time points and placed in heparinized 0.6-ml microcentrifuge tubes. The plasma samples were separated from blood samples by centrifugation at 1000g for 5 min at 4°C. For urine collection, the bladder was rinsed with warm normal saline (2 ml) at the end of each collection. Urine pH and volume were measured. Blank plasma and urine samples were collected before GHB administration and were used to determine endogenous concentrations of GHB. All plasma and urine samples were stored at −80°C until analysis by liquid chromatography-tandem mass spectrometry (LC/MS/MS) to determine GHB concentrations.

Dosing Regimens. Preliminary studies involving 4-h infusions of GHB at various doses were performed to determine the dosing regimens. The preliminary dosing regimens for GHB were based on the pharmacokinetic parameters reported in previous publications (Lettieri and Fung, 1976, 1978a, 1979). The clearance of inulin, used for the determination of glomerular filtration rate (GFR), was also determined after infusions under steady-state conditions. The dose was based on our previous report (Darling and Morris, 1991). Steady-state plasma concentrations of inulin and GHB were obtained by 1 h (data not shown). GHB and inulin were concomitantly administered via an i.v. bolus of a single loading dose of 100, 200, or 400 mg/kg GHB and 60 mg/kg inulin, followed by an i.v. infusion of 108, 128, or 208 mg/h/kg GHB and 100 mg/h/kg inulin for 3 h for low- (n = 4), medium- (n = 4), and high- (n = 5) dose groups. These doses were designed to produce steady-state plasma concentrations of GHB of 200, 400, and 800 mg/l, respectively. Blood samples were collected at 0 (blank), 1, 2 and 3 h, and urine samples at 0 (blank) and between 1 and 3 h. The potential effect of GHB on inulin clearance was determined by administering inulin alone and with GHB.
To examine the effect of various treatments on GHB clearance, GHB was administered at the medium dose (i.v. bolus of 200 mg/kg, followed by 128 mg/h/kg i.v. infusion) in the absence or presence of L-lactate, pyruvate, D-mannitol, sodium bicarbonate, or normal saline (0.9% NaCl) (n = 4 each group). Crossover studies were performed with two 3-h periods (control and treated). Inulin was concomitantly administered during both 3-h periods. L-Lactate, pyruvate, D-mannitol [10% (w/v)] or 0.15 M sodium bicarbonate was administered by an i.v. infusion of 605, 594, 412, or 52 mg/h/kg, respectively; in combination with the i.v. infusion of 128 mg/h/kg GHB and 100 mg/kg/h inulin for 3 h. Blood samples were collected at 0 (blank), 1, 2, 3, 4, 5, and 6 h, and urine samples at 0 (blank), 1 to 3, and 4 to 6 h after administration of GHB.

Protein Binding. Protein binding determinations in rat plasma were performed using ultrafiltration (Centrifree; Millipore, Bedford, MA), as described previously (Morris and Darling, 1990). [3H]GHB with added cold non-radiolabeled GHB was incubated with rat plasma for times up to 4 h, before filtration, but no differences were observed with incubations of 30 min or greater. Concentration-dependent binding studies were performed by adding [3H]GHB, along with cold GHB to produce concentrations of 0.52 to 1000 μg/ml. [3H]GHB in the original sample and ultrafiltrate were determined by scintillation counting.

Determination of Pharmacokinetic Parameters. The total clearance (CL) of GHB was determined from its plasma concentration at steady-state (C_m) and infusion rate (f_d) by the equation of k_d/C_m. Renal clearance (CL_R) was determined by dividing the urinary excretion rate (dA/dt) by the mid-point plasma concentration of GHB (C_m/2). The fraction of the dose eliminated by renal excretion (f_r) was calculated by CL_R/CL. GFR was determined from the inulin (IN) clearance (k_R/IN/Cl_m), where k_R and Cl_m represent the infusion rate and plasma concentration at steady state of inulin. The renal filtration rate of GHB was determined as the product of GFR and the unbound (free) plasma concentration (C_u). where C_u was approximately equal to C_m because GHB is negligibly protein bound in rat plasma. The renal tubular reabsorption rate of GHB was calculated by the difference between renal filtration rate and urinary excretion rate, assuming there is negligible renal secretion of GHB. A lack of renal secretion has been reported for ketone bodies of β-hydroxybutyrate (the congener of GHB with the hydroxyl group at carbon 3) and acetacetate, even at high plasma concentrations of 15 mM (Ferrier et al., 1992). Since both GHB and inulin reached steady state by 1 h, the average steady-state concentrations of 1 to 3 h (or 4–6 h) were used for C_m and Cl_m in the calculations of GFR and renal and total clearances. The metabolic clearance (CL_M) was calculated indirectly from our data using the equation of Cl_m = CL_R – CL_d, assuming that total plasma clearance of GHB is equal to renal clearance plus metabolic clearance. The in vivo Michaeilis-Menten parameters (V_max and K_m) for the nonlinear metabolic clearance were determined by fitting the metabolic clearance values to a Michaelis-Menten equation using nonlinear regression (WinNonlin 2.1; Pharsight, Cary, NC).

LC/MS/MS Assay. The concentration of GHB in rat plasma and urine samples was measured by a validated LC/MS/MS assay that was used for rat brain and plasma samples (Fung et al., 2004). The procedures for sample preparation and data validation for rat plasma and urine samples are briefly described here. The LC/MS/MS system consisted of a PerkinElmer Sciex API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA), equipped with a heated nebulizer interface (PerkinElmer Sciex), a Series 200 PerkinElmer micropump (PerkinElmer, Boston, MA), and a Series 200 PerkinElmer autosampler (PerkinElmer). The separation by reversed-phase high-performance liquid chromatography was performed using an Aqua C18 5-μm 125-Å column (150 × 4.6 mm i.d.; Phenomenex, Torrance, CA) protected by a C18 5-μm guard column cartridge system (Phenomenex). Compounds were eluted isocratically with a mobile phase consisting of 5 mM formic acid/acetonitrile (90:10 (v/v)). The flow rate was 0.9 ml/min and the injection volume was 10 μl. The mass spectrometer was operated in a negative ionization mode with an optimized ion-yield setting for GHB. The interface temperature was set at 400°C. The declustering potential and collision energy for fragmentation were both set at −20 eV. Multiple reaction monitoring was used for specific detection of GHB and GHB-D6 by MS/MS measuring the ion pair transitions of m/z 103 (parent ion) to m/z 57 (product ion) and of m/z 109 (parent ion) to m/z 61 (product ion), respectively. The retention time of GHB and GHB-D6 was 2.6 min, and there were no interfering peaks in plasma and urine samples. The Analyst software version 1.3.1 (Applied Biosystems) was used for instrument control and data analysis.

The lower limit of quantification (LLOQ) was determined during the evaluation of the linear range of the calibration curve. LLOQ was defined as the concentration of the lowest quality control samples producing an assayed concentration within 10% of the theoretical value (i.e., accuracy between 90 and 110%) and yielding a precision of more than 90% for both within- and between-day evaluation. The linearity of calibration curve was evaluated by regression analysis of peak area ratios (GHB/GHB-D6) to GHB concentrations in blank plasma and urine samples, respectively. The accuracy was determined by comparing the calculated concentration using calibration curves of known concentrations. The precision was determined by the coefficient of variation (CV%). Within-day variability was assessed through the analysis of quality controls in triplicate, and between-day variability was determined through the analysis of quality controls on four separate days.

Plasma and Urine Sample Preparation. The stock solutions of 100 μg/ml GHB and internal standard GHB-D6 (5 μg/ml) were freshly prepared. To each blank plasma or urine sample (114 μl; undiluted or appropriately diluted with H2O) was added GHB-D6 stock solution (5 μg/ml; 14 μl) and varying concentrations of GHB (14 μl) to prepare standards of GHB (final concentrations of 100, 200, 500, 1000, 2000, 5000, and 10,000 ng/ml) for the calibration curve. The internal standard GHB-D6 was added in the same concentration and volume to each plasma or urine sample from the animal studies. Solid phase extraction was used with a Bond Elut SAX silica ion exchange column (100 mg; 1 ml) (Varian, Inc., Harbor City, CA), conditioned with 1 ml of methanol, 6 ml (1 ml × 6) of H2O/acetic acid (90:10 (v/v)), and 1 ml of H2O. The plasma or urine samples (100 μl) were loaded on the column and left for 15 min for binding equilbrium. The column was then washed with 0.5 ml of H2O, 0.5 ml of H2O/methanol (50:50 (v/v)), and 0.3 ml of methanol. The column was eluted with 3 ml (1 ml × 3) of acetic acid/acetonitrile (90:10 (v/v)) to obtain the eluents containing GHB and GHB-D6. The eluents were evaporated by N2 gas and reconstituted with 100 μl of H2O or H2O before analysis by LC/MS/MS. Quality control samples of GHB at low (200 ng/ml), medium (1000 ng/ml) and high (5000 ng/ml) concentrations were prepared by the same procedures.

Inulin Assay. The inulin concentrations in plasma and urine samples were determined by a colorimetric method based on the procedure of Davidson and Sackner (1963), with modification. Briefly, a stock solution of inulin (10 mg/ml) was freshly prepared. Inulin (10 μl) was added to blank plasma (10 μl) or urine sample (10 μl; diluted with H2O) to prepare a series of standards of inulin (final concentrations of 5, 10, 20, 40, 60, 80, and 100 μg/ml) for the calibration curve. Each plasma (10 μl) or urine (10 μl; appropriately diluted with H2O) sample from the animal studies was mixed with 50 μl of H2O and 40 μl of trichloroacetic acid (1.0 N). The mixture was allowed to stand for 30 min and centrifuged at 11,000g for 5 min. The supernatant (20 μl) was mixed with 120 μl of anthrone reagent (0.08% (w/v)) that was prepared by dissolving anthrone in H2O/H2SO4 (10:53 (v/v)). The mixture was vortexed for 5 s, chilled on ice for 1 min, and incubated at 38°C in a water bath for 50 min. At the end of the incubation, the mixture was chilled on ice for 2 min and allowed to stand at room temperature for 20 min to equilibrate. The mixture (100 μl) was transferred to a 96-well microplate and the absorbance was determined by microplate spectrometer Spectra Max 340PC (Molecular Devices, Sunnyvale, CA) at 623 nm at 25°C. The
color was stable for as long as 3 h. The assay was validated for sensitivity, linearity, accuracy, and precision based on intraplate and interplate analysis.

**Statistical Analysis.** A one-way analysis of variance, followed by a Dunnett’s post hoc test (Prism 3.0 software; GraphPad Software Inc., San Diego, CA) was used for statistical comparisons involving more than two treatments. Paired Student’s t tests were used for comparing two treatments in the crossover studies. Differences were considered to be significant when $P < 0.05$.

**Results**

**LC/MS/MS Assay for GHB in Plasma and Urine.** The LLOQ was determined as 100 ng/ml. Endogenous concentrations of GHB in plasma and urine were disregarded in this study because their concentrations were less than 1% of those of the GHB concentrations in plasma and urine samples, obtained after the administration of the lowest doses of GHB in our studies. Endogenous plasma values reported by Fung et al. (2004), using the same LC/MS/MS assay, were 2.29 ± 0.19 μg/ml (mean ± S.D.; n = 22), which represent 1% or less of our measured plasma concentrations. Endogenous GHB concentrations in our predose urine samples were 0.28 ± 0.21 μg/ml (mean ± S.D.; n = 9); two other urine samples assayed had GHB concentrations below the LLOQ of the assay due to the dilution of these samples before assay. Bladders were flushed with 2 ml of saline in this study; as well there was further rinsing of the metabolic cages to ensure complete urine collection. Therefore, the values for the urine samples represent the GHB concentrations in these diluted urine samples; the lowest urine concentration we measured in our study was 40 μg/ml.

The calibration curve was linear from 100 to 10,000 ng/ml based on the regression analysis (with the correlation coefficient $r^2 > 0.995$) of peak area ratios (GHB/GHB-D6) versus GHB concentrations. As shown in Table 1, the accuracy for plasma and urine samples was 99 to 114 and 95 to 106%, and the precision expressed as CV% was 1.4 to 10.8 and 1.3 to 13.7% for plasma and urine samples, respectively.

**Protein Binding of GHB.** The protein binding of GHB in rat plasma was found to be low or negligible at GHB concentrations of 0.52 to 1000 μg/ml. The average value determined was 1.2% bound, with no concentration dependence.

**Dose-Dependent Clearance of GHB.** Preliminary studies established that there were no time-dependent differences in inulin clearance when determined over the first 3-h period, or the second 3-h period, during a 6-h infusion. The plasma concentrations of inulin at steady state ($C_{\text{ss,IN}}$) for the low-, medium-, and high-GHB dose groups were not significantly different from that of the control group, demonstrating no effect of GHB on inulin plasma concentrations (Table 2). Correspondingly, there was no significant difference for GFR ($k_{\text{GFR,IN}}C_{\text{ss,IN}}$) values among those groups. These data suggested that the presence of GHB in our studies did not affect the GFR of rats.

Doses of GHB were chosen to produce plasma concentrations of GHB at steady state ($C_{\text{ss}}$) of 0.2, 0.4, and 0.8 mg/ml for the low-, medium-, and high-dose groups, respectively. $C_{\text{ss}}$ values were in these ranges after the low, medium, and high doses (Table 2). From Table 2 and Fig. 2, it can be seen that the renal clearance of GHB, at the doses used in this study, was nonlinear, and the total clearance of GHB was also nonlinear. The renal clearance values increased with increasing GHB doses, whereas the clearance values decreased with increasing GHB doses (Fig. 2). The value of renal clearance for the medium dose was also significantly different from that for the high dose ($P < 0.05$). Since the renal clearance of GHB was much lower than its renal filtration rate, this suggests the presence of renal reabsorption. In fact, the percentages reabsorbed for the medium and high doses were significantly different from that of low dose ($P < 0.001$), decreasing as the dose increased. The metabolic clearance ($C_{\text{m}}$) decreased as the steady-state plasma concentration of GHB increased as indicated in Fig. 2. Using a simple equation $C_{\text{m}} = V_{\text{max}}/(K_{\text{m}} + C_{\text{ss}})$ to fit the data, we obtained a good fit of the data (Fig. 3) with $V_{\text{max}}$ and $K_{\text{m}}$ values of 136 ± 19 mg/h/kg and 0.063 ± 0.047 mg/ml, respectively.

**Clearance of GHB with Modulators.** Studies evaluating the modulators were crossover studies allowing paired comparisons in the same animal for GHB serum concentrations and clearance in the presence and absence of each modulator. The results of the modulator studies are summarized in Table 3. The $C_{\text{ss,IN}}$ and $C_{\text{ss,IN}}$ values were unchanged in the presence of all modulators, indicating no change in GFR. The $C_{\text{ss}}$ values of GHB were significantly ($P < 0.001$) decreased in the presence of L-lactate, pyruvate, D-mannitol, and sodium bicarbonate by 1.6- to 1.8-fold, compared with the crossover control groups (Fig. 4). The renal clearance values of GHB were significantly ($P < 0.001$) increased by 1.8- to 2.9-fold, i.e., 118 ± 20, 132 ± 25, 113 ± 24, and 112 ± 26 ml/h/kg, respectively, compared with the crossover controls (42.7 ± 8.8, 60 ± 11.6, 63.1 ± 12.5, and 60.7 ± 13.9 ml/h/kg, respectively) (Fig. 5). Treatment with normal saline (used as an additional control) produced a small but statistically significant decrease in $C_{\text{ss}}$ but had no effect on the renal clearance of GHB. The renal reabsorption of GHB was significantly ($P < 0.001$) decreased by 1.3- to 1.5-fold, and the total clearance significantly ($P < 0.001$) increased by 1.5- to 1.9-fold by L-lactate, pyruvate, D-mannitol, and sodium bicarbonate treatments (Fig. 6). Normal saline administration did not alter the total clearance of GHB. The metabolic clearance values were significantly increased by treatment groups of L-lactate, pyruvate, and sodium bicarbonate compared with crossover controls; however, there was no significant difference detected for the D-mannitol treatment group. Normal saline treatment did not change the metabolic clearance of GHB. Additional crossover studies were performed adminis-

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**Table 1**

<table>
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<tr>
<th>Matrix</th>
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<th>Accuracy</th>
<th>Precision</th>
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<td>CV%</td>
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tering ammonium chloride 33 mg/kg/h to examine the effects of urine acidification on GHB renal clearance. This study demonstrated no change in the $C_{ss}$, renal clearance, or total clearance of GHB compared with the crossover controls; however, the results of this study have not been included since determination of urinary pH showed no significant acidification of urine samples.

Unpaired comparisons for data obtained over the same time periods can be made by comparing GHB clearance values to those obtained after normal saline administration; these comparisons resulted in similar observations as found with the crossover studies. There were significant differences in the renal and total clearances of GHB after L-lactate, pyruvate, D-mannitol, and sodium bicarbonate treatments. Using normal saline treatment as the control, only L-lactate treatment increased the metabolic clearance of GHB significantly, although the metabolic clearance values increased about 50% for pyruvate, D-mannitol, and sodium bicarbonate.

### TABLE 2

Renal and total clearance of GHB in rats. Studies were performed under steady-state conditions. The results are expressed as mean ± S.D., n = 4 to 5.

<table>
<thead>
<tr>
<th>GHB Dose Group</th>
<th>Inulin Conc. ($C_{\text{ss,IN}}$)</th>
<th>Inulin CL</th>
<th>GHB ($C_{\text{ss}}$)</th>
<th>GHB CLR</th>
<th>GHB Reabsorption</th>
<th>GHB CL</th>
<th>GHB $f_{\text{e}}$</th>
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<tr>
<td>Control</td>
<td>0.15 ± 0.02</td>
<td>617 ± 66.9</td>
<td>0.22 ± 0.04</td>
<td>14.9 ± 5.1</td>
<td>97.5 ± 0.9</td>
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<td>2.9 ± 1.0</td>
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<td>Low</td>
<td>0.18 ± 0.04</td>
<td>646 ± 82.6</td>
<td>0.43 ± 0.05***</td>
<td>51.8 ± 13.0***</td>
<td>92.9 ± 2.2***</td>
<td>302 ± 40**</td>
<td>17.1 ± 3.6***</td>
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<td>Medium</td>
<td>0.16 ± 0.02</td>
<td>695 ± 41.1</td>
<td>0.68 ± 0.11***</td>
<td>97.1 ± 43.1***</td>
<td>83.50 ± 6.3***</td>
<td>312 ± 50.1**</td>
<td>32.1 ± 16.2***</td>
</tr>
<tr>
<td>High</td>
<td>0.18 ± 0.02</td>
<td>593 ± 102</td>
<td>0.43 ± 0.05***</td>
<td>51.8 ± 13.0***</td>
<td>92.9 ± 2.2***</td>
<td>302 ± 40**</td>
<td>17.1 ± 3.6***</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by comparison of the medium- and high-dose groups to the low-dose group; control group is given inulin only.

Fig. 2. Dose-dependent renal clearance (CLR), metabolic clearance ($CL_m$), and total clearance (CL) of GHB. Results are presented as mean ± S.D., n = 3 to 5. Statistical differences were observed compared with the low dose (**, $P < 0.01$; ***, $P < 0.001$), using a one-way analysis of variance followed by a Dunnett's post hoc test.

Fig. 3. Metabolic clearance of GHB at various steady-state plasma concentrations of GHB. The data were fitted using nonlinear regression analysis as stated in the experimental section (●, observed data; —, fitted lines).
TABLE 3

Effects of modulators on the renal and total clearances of GHB in rats

Studies were performed under steady-state conditions. The results are expressed as mean ± S.D., n = 4.

<table>
<thead>
<tr>
<th>GHB Dose Group</th>
<th>Inulin Conc. $C_{\text{ss,IN}}$ (mg/ml)</th>
<th>Inulin CL (ml/h/kg)</th>
<th>GHB Conc. $C_{\text{ss}}$ (mg/ml)</th>
<th>GHB CL (ml/h/kg)</th>
<th>Reabsorption (%)</th>
<th>GHB CL (ml/h/kg)</th>
<th>GHB $f_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHB only</td>
<td>0.20 ± 0.01</td>
<td>511 ± 24</td>
<td>0.37 ± 0.01</td>
<td>63.3 ± 10.0</td>
<td>87.6 ± 2.0</td>
<td>342 ± 5</td>
<td>18.4 ± 2.7</td>
</tr>
<tr>
<td>Normal saline</td>
<td>0.20 ± 0.02</td>
<td>517 ± 40</td>
<td>0.41 ± 0.02</td>
<td>66.0 ± 14.1</td>
<td>87.0 ± 3.9</td>
<td>317 ± 14.7</td>
<td>21.0 ± 5.5</td>
</tr>
<tr>
<td>L-Lactate</td>
<td>0.18 ± 0.01</td>
<td>582 ± 52</td>
<td>0.25 ± 0.03***</td>
<td>118 ± 20***</td>
<td>79.6 ± 3.9</td>
<td>513 ± 65***</td>
<td>23.2 ± 3.9</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.20 ± 0.01</td>
<td>572 ± 30</td>
<td>0.29 ± 0.02***</td>
<td>131 ± 25***</td>
<td>77.1 ± 3.4*</td>
<td>452 ± 38*</td>
<td>29.5 ± 7.4</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>0.22 ± 0.01</td>
<td>529 ± 44</td>
<td>0.27 ± 0.05***</td>
<td>113 ± 24*</td>
<td>78.8 ± 3.2*</td>
<td>474 ± 72*</td>
<td>24.8 ± 8.8</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.18 ± 0.02</td>
<td>637 ± 64</td>
<td>0.28 ± 0.05***</td>
<td>112 ± 26*</td>
<td>82.2 ± 5.5</td>
<td>470 ± 91***</td>
<td>24.3 ± 6.9</td>
</tr>
</tbody>
</table>

* $P < 0.05$.
*** $P < 0.001$.

Fig. 4. Plasma concentrations of GHB obtained in the absence and presence of different treatments. Results are presented as mean ± S.D., n = 4 per group. Control values (black columns) and treatment values (gray columns), * $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$. NS, normal saline.

Fig. 5. Renal clearances of GHB obtained in the absence and presence of different treatments. Results are presented as mean ± S.D., n = 4 per group. Control values (black columns) and treatment values (gray bars), *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$. NS, normal saline.

Urine pH Changes with GHB and Modulators. The urine pH values were stable around pH 6.8 in the absence of drug administration, but they increased after GHB infusion, from 6.54 ± 0.24 at 1 h to 8.27 ± 0.05 at 6 h (Fig. 7A). Administering l-lactate, pyruvate, sodium bicarbonate, and D-mannitol, without GHB, also increased urine pH significantly, with the values ranging from pH 7.57 ± 0.51 for D-mannitol to pH 8.75 ± 0.13 for l-lactate at 4 to 6 h (Fig. 7B). Normal saline administration did not alter urine pH, and pH values were 6.72 ± 0.30. The coadministration of GHB and modulators did not alter the urine pH beyond that observed with GHB alone (Fig. 7C).

Discussion

The clearance of GHB in animals and humans is dose-dependent (Pol et al., 1975; Sneed et al., 1976; Lettieri and Fung, 1978b, 1979; Ferrara et al., 1992, 1996; Palatini et al., 1993; Scharf et al., 1998), with the predominant pathway (more than 90% of the dose) representing metabolism of GHB to CO$_2$ and H$_2$O through the Krebs cycle (Maitre, 1997). Compared with elimination by metabolism, the renal clearance of GHB has been considered a minor elimination pathway. This is true after low doses of GHB, where only 5 to 7% of unchanged GHB is recovered in the urine of rats (Lettieri and Fung, 1976) and <1% in humans (Ferrara et al., 1992). However, in cases of intoxication of GHB and its prodrugs, the renal clearance of GHB seems to be more important for its elimination, and high urinary concentrations of GHB have been reported in humans (Zvosec et al., 2001; Sporer et al., 2003). We have characterized the renal clearance of GHB in this investigation under steady-state conditions, after the administration of three doses. The renal clearance and the
fraction of the dose excreted unchanged in the urine increased with dose, and the calculated fraction reabsorbed decreased. The metabolic clearance of GHB decreased with increasing GHB plasma concentrations. The $K_m$ value, obtained by fitting the metabolic clearance-plasma concentration data to a Michaelis-Menten equation, was similar to the in vivo $K_m$ value of GHB metabolism reported for the brain (Maitre, 1997). However, this value is different from a previous report from Lettieri and Fung (1979), where the $K_m$ value was obtained from fitting the plasma concentration versus time profiles after three different i.v. bolus doses. Briefly our findings suggest that 1) the renal clearance of GHB is significantly increased at higher GHB plasma concentrations, hence playing a more important role in the elimination of GHB after overdoses; and 2) the nonlinear renal clearance may be due to a carrier-mediated renal reabsorption of GHB, which could be saturated at high tubular fluid concentrations of GHB.

GHB is a four-carbon monocarboxylate with a carboxyl group at carbon 1 and a hydroxyl group at carbon 4 ($pK_a = 4.72$) (Nord et al., 1983). Endogenous short-chain monocarboxylates, such as L-lactate and pyruvate, are freely filtered through the renal glomerulus and almost completely reabsorbed in the proximal tubule, predominantly in their ionized form (Poole and Halestrap, 1993). The saturation of the renal reabsorption of some monocarboxylates, e.g., D-lactate, lactate, pyruvate, acetoacetate, and β-hydroxybutyrate, has been well documented in rats (Roth et al., 1982; Barac-Nieto, 1985; Ferrier et al., 1992; Poole and Halestrap, 1993) and rabbits (Nord et al., 1983; Jorgensen and Sheikh, 1985). To our knowledge, there is only one report examining the renal transport of GHB in rabbit brush-border membrane vesicles isolated from the whole cortex, pars convoluta, and pars recta (Jorgensen and Sheikh, 1985). The authors reported that the renal reabsorption of GHB was mediated by two different Na$^+$-dependent electrogenic transport systems in rabbit brush-border membrane vesicles isolated from the whole cortex, i.e., one high-affinity system in the pars recta and a second low-affinity system in the pars convoluta, based on Eadie-Hofstee analysis of their kinetic data (Jorgensen and Sheikh, 1985). From the results of inhibition studies, it was suggested that GHB and other monocarboxylates, such as acetoacetate, β-hydroxybutyrate, and α-hydroxybutyrate, shared common transport systems (Jorgensen and Sheikh, 1985). GHB can inhibit the transport of L-lactate in rabbit brush-border membrane vesicles with an inhibition constant ($K_i$) of 19 mM (Nord et al., 1983). The transport of short-chain monocarboxylates, such as lactate, pyruvate, and butyrate, is specifically catalyzed by a family of proton-linked monocarboxylate transporters (MCT, SLC16, and SLC16A) (Halestrap and Price, 1999). Among the MCT family members, MCT1 is prevalent on the basolateral surface of epithelial cells in the proximal tubules, MCT2 is found on the basolateral surface of epithelial cells in the collecting ducts, and very high levels of MCT6 mRNA are present in human kidney (Halestrap and Price, 1999). GHB can inhibit the transport of pyruvate by both MCT1 and MCT2, suggesting that it may also be a substrate for these transporters (Lin et al., 1998). Recently, SLC5A8, a member of the sodium solute symporters that can transport short-chain fatty acids such as lactate and pyruvate, has been cloned from human colon and found to be highly expressed in kidney (Li et al., 2003; Miyau-
chi et al., 2004). This may represent the transporter responsible for the sodium-dependent uptake of short-chain fatty acids in the kidney. However, the transport of GHB by the MCTs and SLC5A8 has not been characterized.

One of the objectives of our investigations was to identify strategies to increase the renal and total clearance of GHB, for the treatment of GHB overdoses. One strategy that we evaluated was increasing the renal clearance of GHB by inhibiting the active reabsorption of GHB through the use of the short-chain fatty acids, t-lactate, and pyruvate (substrates for both MCTs and SLC5A8; Halestrap and Price, 1999). These compounds were well tolerated and side effects were not observed. Additionally, we evaluated the use of osmotic diuresis and urinary alkalization to decrease the passive reabsorption of GHB, since these approaches have been useful in the treatment of overdoses of other weak acids, such as salicylic acid (pK_a = 3.5) (Prescott et al., 1982) and phenobarbital (pK_a = 7.4) (Costello and Poklis, 1981). All three approaches resulted in an increased renal clearance and total clearance of GHB, and in a significantly decreased plasma concentration of GHB, suggesting that these strategies may be useful in the treatment of GHB overdoses. However, it does not seem likely that alkalization of the urine had significant effects on the renal clearance of GHB. In this investigation, we found that GHB alone could increase the urine pH significantly, from 6.5 to 8.3, compared with the control receiving only inulin; however, this did not result in a change in its renal clearance. The infusion of t-lactate, pyruvate, sodium bicarbonate, and t-mannitol alone also increased the urine pH significantly, but the coadministration of modulators, including sodium bicarbonate, with GHB, did not increase the urine pH greater than that observed with GHB alone. Based on the Henderson-Hasselbalch equation, more than 99% of GHB exists in ionized form as long as urine pH is greater than 6.7, so these changes in pH would have small effects on the overall percentage of GHB present in an ionized state. Ionized GHB would not be passively reabsorbed and may represent the form of GHB that is actively reabsorbed, since MCTs are symporters, cotransporting monocarboxylates with H^+ ions. Becker et al. (2004) have reported that the transport of lactate via MCT1 is significantly increased when MCT1 is coexpressed in oocytes with the sodium-bicarbonate cotransporter: this may be due to the attenuation of the resulting pH changes induced by MCT1-mediated transport of H^+ with lactate. The exact mechanism of the interaction between GHB and sodium bicarbonate remains to be determined.

A very interesting finding from this study was the large increase in total clearance of GHB that resulted from the administration of t-lactate, pyruvate, mannitol, and sodium bicarbonate; this increase is due, only in part, to the increase in renal clearance. Our data suggest that the metabolic clearance was increased in the treated animals, possibly due to the decreased plasma concentration resulting from the increase in renal clearance. Additional mechanisms may also contribute to the changes in metabolic clearance.

In summary, we have characterized the concentration-dependent renal and total clearances of GHB in rats. The non-linear renal clearance is most likely due to the saturable reabsorption of GHB by members of the MCT and/or SLC5A8 transporter families. Three different strategies to increase the renal clearance of an acidic compound were investigated: use of reabsorption inhibitors, osmotic diuresis using d-mannitol, and urinary alkalization using sodium bicarbonate. All three approaches resulted in the decreased renal reabsorption of GHB and an increase in the renal and total clearances: plasma concentrations of GHB were significantly decreased. However, the effects of sodium bicarbonate are likely due to the administration of the compound itself and may not be related to changes in urinary pH. The present study provides proof-of-concept for potential strategies that may be useful in the treatment of GHB overdoses.

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References


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