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γ-hydroxybutyrate (GHB)-Induced Respiratory Depression: Combined Receptor-Transporter Inhibition Therapy for Treatment in GHB Overdose

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Abbreviations: ABEC, area below the effect curve; AUC, area under the plasma concentration-time curve; Cl, clearance; Cl_m, metabolic clearance; Cl_R, renal clearance; E_{max}, maximum pharmacodynamic effect; GHB, γ-hydroxybutyrate; MCT, monocarboxylate transporter; SCH50911, (2*S*)-(+)-5,5-dimethyl-2-morpholineacetic acid; T_d, duration of effect; T_{max}, time of maximum effect

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Abstract:

Overdose of γ-hydroxybutyrate (GHB) frequently results in respiratory depression, occasionally resulting in death; however, little is known about the dose-response relationship or effects of potential overdose treatment strategies on GHB-induced respiratory depression. In these studies, the parameters of respiratory rate, tidal volume, and minute volume were measured using whole-body plethysmography in rats administered GHB. Intravenous doses of 200, 600, and 1500 mg/kg were administered to assess the dose-dependent effects of GHB on respiration. To determine the receptors involved in GHB-induced respiratory depression, a specific $GABA_B$ receptor antagonist, SCH50911 ((2*S*)-(+)-5,5-dimethyl-2-morpholineacetic acid), and a specific $GABA_A$ receptor antagonist, bicuculline, were administered prior to GHB. The potential therapeutic strategies of receptor inhibition and monocarboxylate transporter (MCT) inhibition were assessed by inhibitor administration 5 minutes after GHB. The primary effect of GHB on respiration was a dose-dependent decrease in respiratory rate, accompanied by an increase in tidal volume, resulting in little change in minute volume. Pretreatment with 150 mg/kg SCH50911 completely prevented the decrease in respiratory rate, indicating agonism at $GABA_B$ receptors to be primarily responsible for GHB-induced respiratory depression. Administration of 50 mg/kg SCH50911 after GHB completely reversed the decrease in respiratory rate; lower doses had partial effects. Administration of the MCT inhibitor L-lactate increased GHB renal and total clearance, also improving respiratory rate. Administration of 5 mg/kg SCH50911 + L-lactate further improved respiratory rate compared to the same dose of either agent alone, indicating $GABA_B$ and MCT inhibition, alone and in combination, to represent potential therapeutic strategies for treating GHB-induced respiratory depression.

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Introduction

γ-hydroxybutyate (GHB) is a short-chain fatty acid present endogenously in many human tissues due to production via GABA metabolism (Maitre, 1997). GHB has also recently been identified as a useful therapeutic agent for the treatment of narcolepsy and excessive daytime sleepiness in the form of sodium oxybate (Xyrem®; Jazz Pharmaceuticals, Palo Alto, CA). However, GHB has become more popularly known as a drug of abuse. According to reports from the Drug Abuse Warning Network (DAWN) there have consistently been 1,000-2,000 GHB-related emergency department visits reported annually in the U.S. over the past several years (Substance Abuse and Mental Health Services Administration, 2011). GHB overdose can result in manifestations including sedation, coma, hypothermia, bradycardia, respiratory depression and death (Li et al., 1998; Sporer et al., 2003; Caldicott et al., 2004; Galicia et al., 2011). In a recent report of known GHB-associated fatalities, the most common cause of mortality was cardiorespiratory depression (Zvosec et al., 2011). Respiratory depression with the need for mechanical ventilation is also frequently reported in non-fatal cases of GHB intoxication (Li et al., 1998; Mason and Kerns, 2002; Liechti and Kupferschmidt, 2004).

Although respiratory depression is a common symptom of GHB overdose, neither the dose-dependent effects of GHB on this measure, nor the neurotransmitter receptors involved in GHB-induced respiratory depression, have been investigated. There are several proposed actions of GHB, including i) direct action at $GABA_B$ receptors (Bernasconi et al., 1992) ii) direct action at its own putative GHB receptor (Maitre, 1997) and iii) indirect action at GABA receptors via GABA production/release (Hechler et al., 1997; Gobaille et al., 1999). Although there exists evidence for each of these mechanisms in vitro and/or in vivo, many of the toxicological effects of GHB, including sedation, hypothermia, and fatality, can be attributed to agonism at $GABA_B$ receptors (Carai et al., 2001; Kaupmann et al., 2003; Carai et al., 2005).

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Along with a complex pharmacologic profile, the pharmacokinetics of GHB are also notably complicated. In humans, GHB exhibits dose-dependent pharmacokinetics, even at therapeutic concentrations (Palatini et al., 1993). Rats similarly display nonlinear pharmacokinetics, due to several concentration-dependent processes including saturable oral absorption, saturable metabolism, and saturable renal reabsorption (Lettieri and Fung, 1979; Morris et al., 2005). In both humans and rats, GHB metabolism is the predominant route of elimination at low doses, and renal excretion of unchanged drug is minimal (Lettieri and Fung, 1976; Brenneisen et al., 2004). Although limited information exists in humans at supratherapeutic GHB doses, it has been well-documented in rats that renal clearance becomes an increasingly important route of elimination as GHB doses are increased (Morris et al., 2005). This nonlinear renal clearance can be attributed to a concentration-dependent transport process leading to saturable renal reabsorption, demonstrated in our laboratory to involve the group of transporters known as monocarboxylate transporters (MCTs) (Morris et al., 2005; Wang et al., 2006). MCTs are proton-dependent transporters expressed throughout the body, of which GHB is an identified substrate for MCTs 1, 2, and 4 (Wang et al., 2006; Wang and Morris, 2007). The ubiquitous expression of these transporters includes that in the intestine, kidney, and brain, regions of interest regarding GHB pharmacokinetics. Due to their role in the renal reabsorption of GHB, inhibition of these transporters represents a potential therapeutic strategy for GHB overdose. This strategy has been demonstrated to translate to in vivo effects on GHB disposition, as administration of MCT inhibitors increases the renal and total clearance in animal models of GHB overdose (Morris et al., 2005; Wang et al., 2008a; Wang et al., 2008b). Similarly, the administration of the MCT inhibitor L-lactate, in combination with osmotic diuresis, increases the renal clearance of GHB in humans, as demonstrated in our pilot clinical study (Morris et al., 2011).

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The first aim of this research was to investigate the dose-response relationship of GHB-induced respiratory depression, including the primary neurotransmitter receptors involved in eliciting this effect. The second was to assess potential treatment strategies, including MCT and receptor inhibition, for improving GHB-induced respiratory depression, as the application of these strategies for treating this pharmacodynamic endpoint have not been previously evaluated.

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Materials and Methods

Chemicals and Reagents. Sodium GHB used in these studies was provided by the National Institute on Drug Abuse (NIDA). Deuterated GHB (GHB-d_6) was purchased from Cerilliant (Round Rock, TX). Sodium L-lactate and bicuculline methiodide were purchased from Sigma-Aldrich (St. Louis, MO). SCH50911 was purchased from Tocris Bioscience (Minneapolis, MN). HPLC grade acetonitrile and acetic acid were purchased from Honeywell Burdick & Jackson (Muskegon, MI).

Animals and Animal Surgery. Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 270-330 g were used for all experiments. Animals were housed under controlled temperature and humidity with an artificial 12 hour light/dark cycle and food was available *ad libitum*. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University at Buffalo. Animals were allowed to acclimate to their environment for a minimum of one week prior to surgical implantation of jugular and femoral vein cannulae under anesthesia with ketamine/xylazine. Cannulae were flushed daily with 40 IU/mL heparinized saline to maintain patency. Animals were allowed a minimum of 72 hours for recovery from surgery before drug administration.

Plethysmography. Measurement of respiration in these studies was performed using a whole-body plethysmograph (Model PLY4213, Buxco Research Systems, Wilmington, NC). Plethysmography equipment included unrestrained plethysmography chambers consisting of a main (animal) chamber and reference chamber for buffering changes in atmospheric pressure. The plethysmography chambers were connected to a Rodent Bias Flow Supply (BFL0250) to draw expired CO_2 out of the chambers and provide a smoothed flow of room air at a flow rate of 2.5 L/min per chamber. The plethysmography chambers included ports to which a pressure sensor was connected and led to the MAX 1500 preamplifier. Signals were collected, visualized, and quantitated using BioSystem

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XA Software. Two additional ports were included in the chambers for the insertion of jugular and femoral vein cannulae, allowing for drug administration and blood sampling. Urine was collected at the base of the chamber and collected at intervals by opening an additional port at the base. Calibration of chamber pressure was performed prior to every experiment by injection of 5 mL air through the base port. At each recording, signals were collected for 6 intervals of 10 seconds each, and averaged to represent 1 minute of recording. Measurements for the parameters of respiratory frequency (rate), tidal volume, and minute volume (rate · tidal volume) were quantitated for each recording.

Pharmacokinetic/Pharmacodynamic Studies. Rats were placed in plethysmography chambers 1 hour before drug administration and allowed to acclimate to the chambers for 45 minutes before 5 baseline measurements of 1 minute each were collected over 15 minutes. In all studies, GHB administration was considered time 0 and respiration measurements were recorded at 2.5, 5, 7.5, 10, 15, 20, 25, and 30 minutes and every 15 minutes thereafter for 480 minutes. Blood samples were collected and collection times optimized for each GHB dose according to previous studies (Felmlee et al., 2010a; Felmlee et al., 2011). Urine was collected at intervals up to 480 minutes. For overlapping pharmacokinetic/pharmacodynamic time points, blood and urine samples were taken directly after the recording of respiratory measurements.

Dose-dependent effects of GHB on respiration. To assess the dose-response relationship of GHB-induced respiratory depression, rats were administered GHB intravenously in doses of 200, 600, and 1500 mg/kg, (4-6 animals/dose). GHB was injected over 1-2 minutes as a 300 mg/mL solution in sterile water via the jugular vein cannula. A placebo control group received a 5 mL/kg saline bolus.

Neurotransmitter receptors involved in GHB-induced respiratory depression. To determine the primary receptors involved in GHB-induced respiratory depression, rats

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were pre-treated with specific receptor antagonists. Bicuculline methiodide (5 mg/kg) was administered for inhibition of $GABA_A$ receptors and SCH50911 (150 mg/kg) for inhibition of $GABA_B$ receptors, (3-4 animals/group). Inhibitors were administered immediately after the collection of baseline respiratory measurements and GHB 1500 mg/kg administered 5 minutes later. Data from dose-dependent experiments were used as control. Bicuculline methiodide was administered as a 5 mg/mL solution in saline and SCH50911 as a 50 mg/mL solution in saline via the jugular vein cannula.

Potential treatment strategies. To assess the effect of potential treatment strategies on GHB-induced respiratory depression, treatments were administered intravenously 5 minutes after GHB 1500 mg/kg. Treatment strategies included SCH50911 (2.5, 5, 10, and 50 mg/kg), the MCT inhibitor L-lactate (66 mg/kg bolus followed by a 302.5 mg/kg/hr infusion for 8 hours), and combination therapy of 5 mg/kg SCH50911 + the same dose of L-lactate. Treatment groups included 3-5 animals/group, and were compared to the 1500 mg/kg control group from dose-dependent experiments to determine effects of treatment on GHB-induced respiratory depression. The same L-lactate dose was also administered alone at time 0 to assess potential effects of this agent on respiration. In these experiments, SCH50911 was administered as a 2.5, 5, 10, or 50 mg/mL solution in saline via the jugular vein cannula and L-lactate as a 40 mg/mL solution in sterile water via the femoral vein cannula.

Plasma and Urine Sample Analysis. GHB plasma concentrations were determined using an LC/MS/MS method, similar to those published previously (Fung et al., 2008; Felmlee et al., 2010b). Plasma samples were prepared by adding 5 µl of GHB-d_6 (125 µg/ml) to 50 µl of sample. Plasma standards and quality controls were prepared by adding 5 µl of GHB-d_6 and 5 µl of GHB stock solution to 45 µl of blank plasma. 800 µl of 0.1% formic acid in acetonitrile was added to precipitate the plasma proteins. The

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samples were vortexed followed by centrifugation at 10,000g for 20 minutes at 4^{o}C. 750 µl of the supernatant was aspirated and then evaporated under a stream of nitrogen gas. The samples were reconstituted in 250 µl of aqueous mobile phase.

The LC/MS/MS assay was performed on Agilent 1100 series HPLC with binary pump and autosampler (Agilent Technologies, Santa Clara, CA) connected to a Perkin Elmer Sciex API 3000 triple-quadrupole tandem mass spectrometer with a turbo ion spray (Applied Biosystems, Foster City, CA). Chromatographic separation was achieved by injecting 7 µl of sample on an Xterra MS C18 column (250 x 2.1 mm i.d., 5-µm particle size; Waters, Milford, MA). Mobile phase A consisted of 5/95 acetonitrile/water with 0.1% acetic acid and mobile phase B of 95/5 acetonitrile/water with 0.1% acetic acid. The flow rate was 200 µl/min with the following gradient elution profile: 100% to 68% A over 7 min; 68% to 10% A over 3 min, and 10% to 100% over 5 min for a total run time of 15 min. The mass spectrometer was operated in a positive ionization mode with multiple reaction monitoring. Q1/Q3 m/z ratios for the parent/product ions of GHB and GHB-d_6 were 105.2/87.2 and 111.1/93.2, respectively. The mass spectrometer parameters were optimized at a declustering potential of 18 V, focusing potential of 100 V, collision energy of 20 V, entrance potential of 10 V, and collision cell exit potential of 5 V. The ion spray voltage was set at 5500 V with temperature at 350^{o}C. Nebulizer and curtain gas flow were set at 10 ml/min and 8ml/min, respectively. The retention time for GHB was 4.15 minutes. The data were analyzed using Analyst software version 1.4.2 (Applied Biosystems, Foster City, CA).

Regression analysis of peak area ratios of GHB/GHB-d_6 to GHB concentrations was utilized to assess linearity of the curve. The intra-day and inter-day precision and accuracy were determined using quality control samples at 10 µg/ml (low QC), 125 µg/ml (medium QC) and 400 µg/ml (high QC). For determination of the intra-day

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precision and accuracy, quality control samples were analyzed in triplicate on each day whereas for the inter-day precision and accuracy, quality control samples were analyzed on three different days. A calibration curve was run on each analysis day along with the quality controls. The precision was determined by the coefficient of variation (CV%) and accuracy was measured by comparing the calculated concentration to the known concentration.

Urine samples were prepared and analyzed for GHB using a previously described LC/MS/MS method (Felmlee et al., 2010a). Plasma lactate concentrations were determined using a YSI 1500 Sport Lactate Analyzer (Yellow Springs Instruments, Inc., Yellow Springs, OH).

Data and Statistical Analysis. Pharmacokinetic parameters were determined via noncompartmental analysis using WinNonLin 5.2 (Pharsight Inc., Palo Alto, CA). Area below the plasma concentration-time curve (AUC) was determined using the trapezoidal method. Total clearance (Cl) was determined as Dose/AUC. Renal clearance (Cl_R) was determined as A_e/AUC, where A_e represents the amount excreted in the urine. Percentage of urinary excretion was calculated as A_e/Dose. Metabolic or nonrenal clearance (Cl_m) was calculated as Cl-Cl_R. The pharmacodynamic descriptors of area below the effect curve (ABEC), maximum effect (E_{max}), time of maximum effect (T_{max}), and duration of effect (T_d) were used to determine the effects of inhibitor administration on GHB-induced respiratory depression. ABEC was calculated using WinNonLin. T_d was determined for each animal as the time to return to its individual baseline respiratory frequency. Statistical analysis was performed using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA). Differences were considered significant when $p<0.05$. One-way analysis of variance followed by Dunnett's or Tukey's posthoc tests were used to determine statistically significant differences in mean pharmacokinetic and pharmacodynamic parameters between groups. Paired t-tests were used to determine

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statistically significant changes in respiratory parameters compared to baseline. In determining the effects of L-lactate alone on respiration, the average of the last hour of respiratory measurements was compared to the individual average baseline values. Mean steady-state lactate plasma concentrations were calculated as the average of hourly values beginning at 60 minutes.

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Results

Plasma GHB LC/MS/MS assay. The lower limit of quantification (LOQ) for GHB in plasma was found to be 5 μg/ml with acceptable error in precision and accuracy of less than 20%. The endogenous concentrations of GHB in plasma are negligible when compared to GHB concentrations obtained after administration of the lowest dose in our studies (Fung et al., 2004); therefore, the endogenous concentrations were not included in the calculation of GHB concentrations in plasma. The standard curve for GHB ranged from 5-500 μg/ml based on regression analysis of peak area ratios of GHB/GHB-d_6 to GHB concentrations with a correlation coefficient, r^2>0.999. The intra-day and inter-day precision and accuracy of the quality control samples are summarized in Table 1.

Dose-dependence of GHB pharmacokinetics/pharmacodynamics. GHB administration in increasing intravenous doses displayed nonlinear pharmacokinetics, shown in Table 2, similar to previous reports (Lettieri and Fung, 1979; Morris et al., 2005). Renal clearance and the urinary excretion of GHB was almost negligible at the lowest dose of 200 mg/kg, but represented the predominant route of elimination at the highest dose of 1500 mg/kg. The pharmacodynamic results of this experiment are shown in Figure 1. Increasing doses of GHB resulted in a dose-dependent decrease in the parameter of respiratory rate, which was accompanied by a dose-dependent increase in tidal volume. Minute volume was unchanged with the 200 and 600 mg/kg doses, but was significantly decreased with the 1500 mg/kg dose (95 ± 18 mL/min at baseline vs. E_{max} of 54 ± 24 mL/min; mean±SD, $p<0.05$). Raw plethysmography traces displaying the change in respiratory pattern with GHB administration are shown in Figure 2. Due to the results of this experiment, respiratory rate was considered the primary parameter of interest for assessment of receptors involved and potential treatment strategies. It was also determined in this experiment that GHB 1500 mg/kg was the maximum dose that could

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be administered without resulting in death, therefore this dose was used for further investigation.

Neurotransmitter receptors involved in GHB-induced respiratory depression. Effects of pretreatment with specific receptor antagonists are given in Table 3. Administration of the $GABA_B$ inhibitor, SCH50911 (150 mg/kg), prior to GHB, resulted in no significant decrease in respiratory rate, nor a change in tidal volume compared to baseline, as displayed in Figure 3. This inhibitor also increased the nonrenal clearance of GHB, but not the total clearance at this dose. Administration of the $GABA_A$ inhibitor, bicuculline methiodide (5 mg/kg), prior to GHB, resulted in no change in the respiratory effects compared to GHB alone and had no significant effects on GHB pharmacokinetics.

Potential treatment strategies. Effects of potential treatment strategies on GHB-induced respiratory depression are given in Table 4. Administration of 50 mg/kg SCH50911 five minutes after GHB completely reversed the GHB-induced decrease in respiratory rate, as shown in Figure 4; there was no significant decrease in respiratory rate compared to baseline following the administration of SCH50911. In fact, a slight, but significant increase in respiratory rate was observed at early time points in SCH50911-treated animals. Lower doses of 2.5, 5, and 10 mg/kg SCH50911 did not completely reverse GHB-induced respiratory depression, as significant decreases in respiratory rate were still observed after antagonist administration. Administration of 10 mg/kg SCH50911 significantly improved all pharmacodynamic parameters, whereas 5 mg/kg improved only the ABEC and E_{max}, and 2.5 mg/kg had no significant effect on any pharmacodynamic parameter compared to GHB alone. Administration of 50 mg/kg SCH50911 also increased the nonrenal clearance of GHB, similar to the higher dose of this receptor antagonist, but this effect was not observed at lower doses. Administration of the MCT inhibitor, L-lactate, significantly increased GHB renal and total clearance and resulted in significant decreases in the frequency ABEC and T_d, but did not improve the

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E_{max}. The combined administration of 5 mg/kg SCH50911 + L-lactate improved all pharmacodynamic endpoints compared to GHB alone. When L-lactate was administered alone at the same dose as that given with GHB, the plasma lactate concentrations obtained with this dose were much lower in the absence of GHB, indicating an effect of GHB on lactate pharmacokinetics. A higher dose of 66 mg/kg + 605 mg/kg/hr L-lactate was then administered alone to achieve similar lactate concentrations as obtained with GHB administration; ~1.5 mM mean increases in plasma lactate concentrations were obtained with this higher dose and with the lower dose administered concomitantly with GHB, as shown in Figure 5. As displayed in Figure 6, this higher dose of L-lactate had no significant effect on respiratory rate, tidal volume, or minute volume.

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Discussion

Although abuse of GHB and its precursors has been recognized, there still exists no pharmacologic treatment for GHB overdose and current treatment consists of supportive care. Due to high rates of respiratory depression reported in both fatal and non-fatal GHB overdose cases, this pharmacodynamic endpoint serves as a clinically relevant marker for the evaluation of potential GHB overdose treatment strategies. The current data indicate the primary effect of GHB on respiration to be a decrease in respiratory rate, accompanied by a compensatory increase in tidal volume, allowing minute volume to remain constant until doses approach lethality. This respiratory pattern is similar to that noted in some clinical cases of GHB overdose (Mason and Kerns, 2002), indicating our rat model to be relevant for studying this endpoint. When the respiratory rate decreases substantially, and tidal volume reaches a physiologic limit, minute volume decreases steeply with increases in GHB concentrations resulting in fatality. Although we observed no fatalities at doses of 1500 mg/kg IV and lower, we observed fatality in approximately 50% of animals administered 1750 mg/kg, consistent with previous reports indicating the LD_{50} of GHB administered intravenously in rats to be 1700 mg/kg (Laborit, 1964). These results indicate minute volume to be an insensitive measure of GHB intoxication, due to little change in this measure before death, and the primary parameter of interest in GHB-induced respiratory depression to be respiratory rate.

Our data indicate the decrease in respiratory rate following GHB administration to be mediated primarily by action at $GABA_B$ receptors, due to complete inhibition of the effect of GHB on this parameter with pretreatment of a $GABA_B$ receptor antagonist, SCH50911. This inhibitor also exhibited a surprising effect on GHB pharmacokinetics, significantly increasing its nonrenal clearance. This effect was likely not translated to increased total clearance since nonrenal clearance is a minor route of GHB elimination

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at 1500 mg/kg, but may be responsible for some of the effects of this inhibitor observed on GHB toxicodynamics in studies at lower GHB doses. Although the primary mechanism of GHB-induced respiratory depression was evident from $GABA_B$ receptor antagonism, we sought to assess any contribution of GABA on the effects of GHB by administration of bicuculline, a $GABA_A$ receptor antagonist. Bicuculline pretreatment did not significantly affect any of the pharmacodynamic descriptors for frequency or tidal volume, compared to GHB alone, indicating a negligible effect of GABA agonism at $GABA_A$ receptors on GHB-induced respiratory depression. We did not assess the effects of GHB receptor antagonism in these studies, since previous publications indicate minimal, or even protective effects of GHB at this receptor with regards to toxicodynamic endpoints (Carai et al., 2001; Carai et al., 2005). Due to results indicating the role of $GABA_B$ receptors in GHB-induced respiratory depression, SCH50911 was also administered after GHB to assess the potential of $GABA_B$ inhibition as a treatment strategy for reversing GHB-induced respiratory depression. Similar to other reports (Carai et al., 2001; Carai et al., 2005), complete reversal of this toxicodynamic endpoint required a high dose of this inhibitor, 50 mg/kg, although significant effects were observed at doses as low as 5 mg/kg. Previous studies have assessed the effect of SCH50911 alone on respiration, reporting no effect at doses up to 100 mg/kg (Bolser et al., 1995).

Although the administration of $GABA_B$ antagonists has the capability of completely preventing/reversing GHB-induced respiratory depression, these agents are not currently available for human use. Additionally, complete reversal of GHB's toxicological effects requires large doses of these inhibitors. In contrast, MCT inhibition with L-lactate represents a practical treatment strategy due to its clinical availability (Lactated Ringers Solution, Lactate for Injection USP) and demonstrated safety and efficacy for increasing GHB renal clearance in humans at relevant clinical dosages

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(Morris et al., 2011). The dose of L-lactate administered in this study was given to target a 1-2 mM increase in plasma lactate concentrations, the same range targeted in our clinical study. Higher L-lactate regimens were also administered in the current study, but with no greater effect. When administered at 5 minutes after GHB, L-lactate significantly increased GHB total and renal clearances, resulting in significant improvement of the frequency ABEC and T_d. A significant decrease in respiratory rate was still observed, however, with no improvement in the E_{max}. It was hypothesized that further improvement in respiratory rate may be obtained by combination therapy including a low dose $GABA_B$ antagonist and L-lactate, due to the differing mechanisms and partial, but temporally distinct effects on GHB-induced respiratory depression. We administered SCH50911 at the lowest effective dose of 5 mg/kg with L-lactate, and as the results of these experiments demonstrate, this combination therapy resulted in significant improvement of all pharmacodynamic parameters, which was not achieved with the same doses of these inhibitors alone. A pertinent effect of L-lactate, with and without SCH50911, is the improvement in the duration of respiratory depression, T_d. A need for intubation with mechanical ventilation is frequently reported in overdose cases of GHB, even when respiratory depression does not result in fatality, and improvement in T_d likely indicates improvement in the duration of intubation. This parameter may be of particular concern following oral overdose of GHB and its precursors, due to the prolonged high GHB plasma concentrations observed after oral ingestion of these agents at high doses (Lettieri and Fung, 1978; Lettieri and Fung, 1979; Fung et al., 2008). Another interesting effect of SCH50911/L-lactate combination was an increased GHB clearance compared to L-lactate alone. Along with the improvement in pharmacodynamic parameters, this increased clearance of GHB may represent an added benefit of combination therapy compared to either single agent. Altogether, these data suggest L-lactate administration to be effective for treating GHB-induced respiratory depression, with and without $GABA_B$

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antagonists. Further studies will need to include the assessment of treatment strategies in oral GHB overdose and the efficacy of treatments administered at various times following the GHB overdose. Also of importance is the assessment of respiratory depression in GHB overdose when concomitantly administered with other drugs of abuse, particularly ethanol and opiates, which themselves can affect respiration.

Due to reports indicating both stimulatory and inhibitory effects of L-lactate on respiration (Gorman et al., 1988; Tappy et al., 1996; Olsson et al., 2002), the dose of L-lactate administered concomitantly with GHB in these studies was administered alone to determine the effects of our targeted lactate plasma concentrations on respiration. Although an effect of L-lactate on GHB pharmacokinetics has been well-described (Morris et al., 2005; Wang et al., 2006; Wang et al., 2008a), an interesting observation in this study was an apparent effect of GHB on L-lactate pharmacokinetics. Much lower plasma lactate concentrations were observed when the same dose of L-lactate was administered alone compared to those achieved with GHB co-administration indicating inhibition of lactate clearance by GHB. Urinary recovery of lactate was increased with GHB administration, compared to L-lactate alone, likely due to inhibition of renal MCT-mediated reabsorption (0.055 ± 0.015% vs. 1.4 ± 0.41% total dose; mean ± SD, $p<0.01$). This effect was not translated in the plasma profiles due to the negligible role of renal clearance in total lactate elimination, at this dose. The decrease in lactate clearance, therefore, indicates a decrease in L-lactate metabolism with GHB administration. This effect is not entirely unexpected considering hepatic lactate metabolism has been demonstrated to be uptake rate-limited (Metcalfe et al., 1986). Administration of β-hydroxybutyrate, an MCT substrate with similar affinity as that of GHB, significantly inhibited lactate uptake and metabolism in hepatocytes and a rat liver perfusion model at a concentration of 10 mM (Metcalfe et al., 1986). Concentrations of GHB in the current studies reach 40 mM with the 1500 mg/kg dose, and are therefore likely high enough to

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significantly inhibit lactate transport and metabolism. Due to the observed pharmacokinetic interaction, a higher dose of L-lactate was administered to achieve the same targeted 1-2 mM increase in plasma concentrations. This dose resulted in no significant change in any respiratory parameter, although an insignificant 16% mean decrease in minute volume was observed. The lack of significant effect in our study is likely due to lower plasma lactate concentrations obtained compared to others which report effects of L-lactate on respiration, and indicates no adverse effects of our targeted concentrations.

In summary, GHB overdose leads to respiratory depression due to a decrease in respiratory rate, and similar to other toxicological effects of GHB, this effect is mediated primarily by agonism at $GABA_B$ receptors. Increasing GHB clearance via MCT inhibition with L-lactate improves GHB-induced respiratory depression, and the clinical availability of this treatment option makes this a practical therapeutic strategy. A novel treatment strategy including combination therapy of a low dose $GABA_B$ antagonist and L-lactate may represent a safe and effective GHB overdose treatment strategy, pending the availability of $GABA_B$ receptor antagonists for clinical use. Further studies are necessary to assess the efficacy of treatment strategies including L-lactate following the oral overdose of GHB and its precursors, alone and with other drugs of abuse.

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Authorship Contributions

Participated in research design: Morris and Morse

Conducted experiments: Morse

Contributed new reagents or analytic tools: Morris, Morse, and Vijay

Performed data analysis: Morse

Wrote or contributed to the writing of the manuscript: Morris, Morse, and Vijay

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Footnotes

a) This work was supported by the National Institutes of Health National Institute on Drug Abuse [grant DA023223] and by a fellowship from Pfizer Global Research and Development.

b) A portion of this work was previously presented as an abstract: Respiratory Depression in γ-Hydroxybutyrate Overdose: Interaction with Ethanol and Treatment using Monocarboxylate Transporter Inhibition, American Association of Pharmaceutical Sciences Annual Meeting 2011, Washington DC.

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Figure Legends

Figure 1. Dose-dependent effects of GHB on measures of respiration. GHB was administered intravenously at time 0. Data presented as mean ± SD, n=4-6.

Figure 2. Effect of GHB administration on respiratory pattern. Displayed are sample 10-second interval plethysmography traces obtained A) at baseline and B) 30 minutes after administration of GHB 1500 mg/kg intravenously.

Figure 3. Effect of specific receptor inhibitors on GHB-induced respiratory depression. GHB 1500 mg/kg was administered intravenously, alone and after pretreatment of the $GABA_B$ receptor antagonist, SCH50911 (150 mg/kg), and the $GABA_A$ receptor antagonist, bicuculline methiodide (5 mg/kg). Inhibitors were administered intravenously 5 minutes prior to GHB. Data presented as mean ± SD, n=3-5.

Figure 4. Effect of potential treatment strategies on respiratory rate following GHB administration. A) Dose-dependent effects of SCH50911 B) Effects of L-lactate and L-lactate/SCH50911 combination therapy. All treatment strategies were administered intravenously 5 minutes after GHB 1500 mg/kg intravenously. Data presented as mean ± SD, n=3-5.

Figure 5. Plasma lactate concentrations after administration of L-lactate alone and with GHB. Data presented as mean ± SD, n=4-5. Low dose L-lactate (LD) = 66 mg/kg + 302.5 mg/kg/hr. High dose L-lactate (HD) = 66 mg/kg + 605 mg/kg/hr.
L-lactate LD and HD were administered alone at time 0. L-lactate LD was administered 5 minutes after GHB when administered concomitantly.

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Figure 6. Effect of L-lactate on respiration. Data presented as mean ± SD, n=4. High dose L-lactate (HD) = 66 mg/kg + 605 mg/kg/hr, administered at time 0.

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Table 1. Intra-day and inter-day accuracy and precision for GHB in rat plasma.

	Nominal concentration (μg/ml)	Measured concentration (μg/ml)	SD	Precision (CV%)	Accuracy (%)
Intra-day	10	10.8	0.12	1.07	107.7
	125	121	4.36	3.60	96.8
	400	375	6.93	1.85	93.7
Inter-day	10	10.5	0.32	3.05	105.2
	125	118	2.96	2.50	94.9
	400	368	6.38	1.73	92.0

Each measured concentration is the mean of triplicate measurements. The analysis was performed over 3 days.

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Table 2. Nonlinear pharmacokinetics of GHB

	200 mg/kg	600 mg/kg	1500 mg/kg
Cl (ml/kg/min)	7.60 (0.29)	6.00 (0.74) [a]	5.16 (0.70) [a]
Cl_R (ml/kg/min)	0.444 (0.20)	1.68 (0.75) [a]	3.18 (0.66) [a,b]
% urinary excretion	6.0 (3) %	26.7 (11) % [a]	60.1 (7) % [a,b]

Cl=clearance Cl_R=renal clearance

GHB was administered intravenously. Data presented as mean (SD), n=4-6. One-way ANOVA followed by Tukey's post-hoc test was used to determine statistically significant differences in pharmacokinetic parameters.

[a] significantly different from GHB 200 mg/kg ($p<0.05$)

[b] significantly different from GHB 200 and 600 mg/kg ($p<0.05$)

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Table 3. Effects of specific receptor antagonists on the pharmacokinetics/pharmacodynamics of GHB (1500 mg/kg IV)

	GHB	GHB + SCH50911	GHB + bicuculline
Cl (ml/kg/min)	5.16 (0.70)	6.07 (0.47)	5.02 (0.14)
Cl_R (ml/kg/min)	3.18 (0.66)	2.84 (0.32)	2.72 (0.78)
Cl_m (ml/kg/min)	1.99 (0.17)	3.23 (0.78) *	2.30 (0.63)
Frequency ABEC (breaths)	10500 (2700)	------	10900 (1300)
Frequency E_{max} (breaths/min)	17 (7)	------	15 (9)
Frequency T_{max} (min)	53.0 (19)	------	67.5 (13)

IV=intravenously Cl=total clearance Cl_R=renal clearance Cl_m=metabolic clearance ABEC=area below the effect curve E_{max}=maximum pharmacodynamic effect T_{max}=time of maximum effect

SCH50911 (150 mg/kg) and bicuculline methiodide (5 mg/kg) were administered intravenously 5 minutes prior to GHB. Data presented as mean (SD), n=3-5. One-way ANOVA followed by Dunnett's post-hoc test was used to determine statistically significant differences in mean pharmacokinetic and pharmacodynamic parameters with inhibitor administration compared to GHB alone.

*significantly different from GHB alone ($p<0.05$)

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------ no ABEC, E_{max}, or T_{max} values could be calculated since respiration is similar to the baseline values; SCH50911 completely prevented any significant decrease in frequency compared to baseline

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Table 4. Effects of potential treatment strategies on the pharmacokinetics/pharmacodynamics of GHB (1500 mg/kg IV)

	Control	SCH50911 50 mg/kg	SCH50911 10 mg/kg	SCH50911 5 mg/kg	SCH50911 2.5 mg/kg	L-lactate	SCH50911 5 mg/kg + L-lactate
Cl (mL/kg/min)	5.16 (0.70)	6.17 (0.41)	6.13 (0.22)	6.13 (0.23)	6.05 (0.69)	6.40 [a] (0.62)	7.61 [a,b,c] (0.062)
Cl_R (mL/kg/min)	3.18 (0.66)	3.37 (0.038)	4.03 (0.21)	3.78 (0.36)	3.56 (0.50)	4.22 [a] (0.63)	5.28 [a,b] (0.42)
Cl_m (mL/kg/min)	1.99 (0.17)	2.80 [a] (0.52)	2.09 (0.37)	2.35 (0.17)	2.50 (0.19)	2.19 (0.55)	2.33 (0.44)
Frequency ABEC (breaths)	10500 (2700)	------	3690 [a] (1440)	5500 [a] (1440)	8720 (513)	5470 [a] (1550)	3170 [a] (957)
Frequency E_{max} (breaths/min)	17 (7)	------	51 (3) [a]	44 (6) [a]	33 (2)	24 (5)	45 (6) [a]
T_d (hr)	4.35 (1.3)	------	2.50 [a] (0.20)	3.15 (0.28)	4.62 (1.2)	2.45 [a] (0.62)	2.17 [a] (0.14)

Cl=total clearance Cl_R=renal clearance Cl_m=metabolic clearance

ABEC=area below the effect curve E_{max}=maximum pharmacodynamic effect

T_d=duration of respiratory depression

Data presented as mean (SD), n=3-5. Control=administration of GHB 1500 mg/kg intravenously. SCH50911 and L-lactate were administered intravenously 5 minutes after GHB. L-lactate was administered as a 66 mg/kg bolus followed by a 302.5 mg/kg/hr infusion for 8 hours. One-way ANOVA followed by Tukey's post-hoc test was used to

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detect statistically significant differences in mean pharmacokinetic and pharmacodynamic parameters.

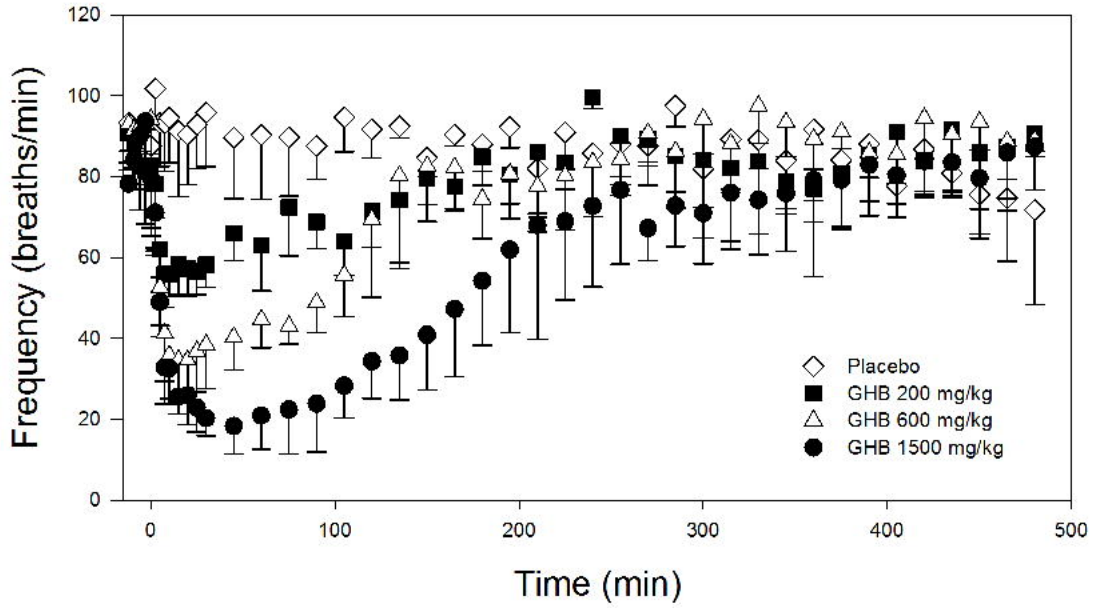
------ no ABEC, E_{max}, or T_d values could be calculated since respiration is similar to the baseline values; no significant decrease in frequency compared to baseline was observed after administration of 50 mg/kg SCH50911

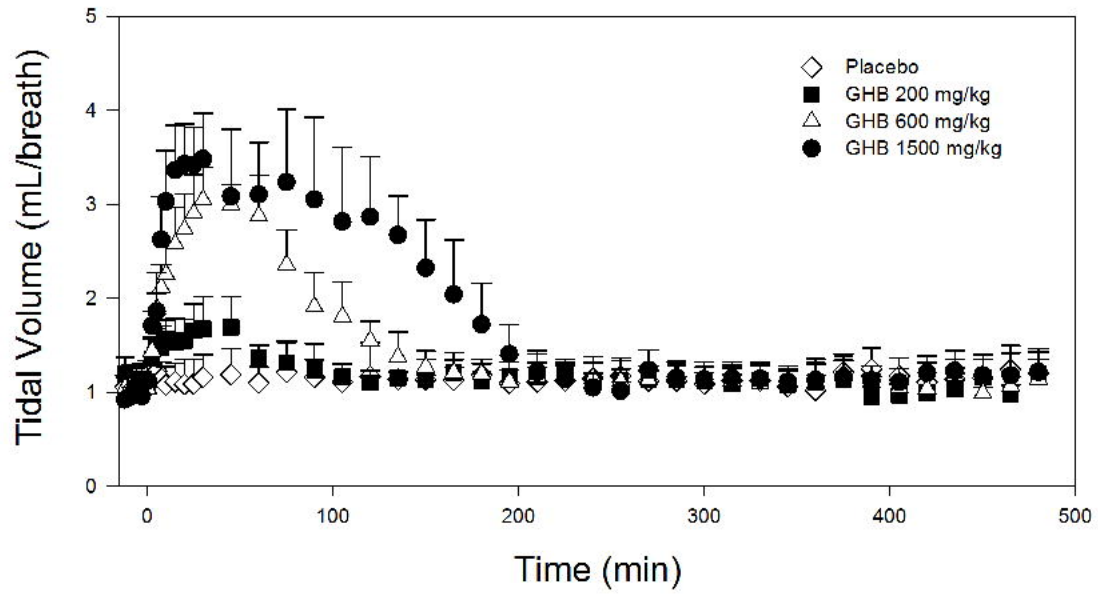
[a] significantly different from control ($p<0.05$)

[b] significantly different from 5 mg/kg SCH50911 alone ($p<0.05$)

[c] significantly different from L-lactate alone ($p<0.05$)

Fig. 1


Frequency (breaths/min)
Time (min)
Placebo
GHB 200 mg/kg
GHB 600 mg/kg
GHB 1500 mg/kg


Tidal Volume (mL/breath)
Time (min)
Placebo
GHB 200 mg/kg
GHB 600 mg/kg
GHB 1500 mg/kg

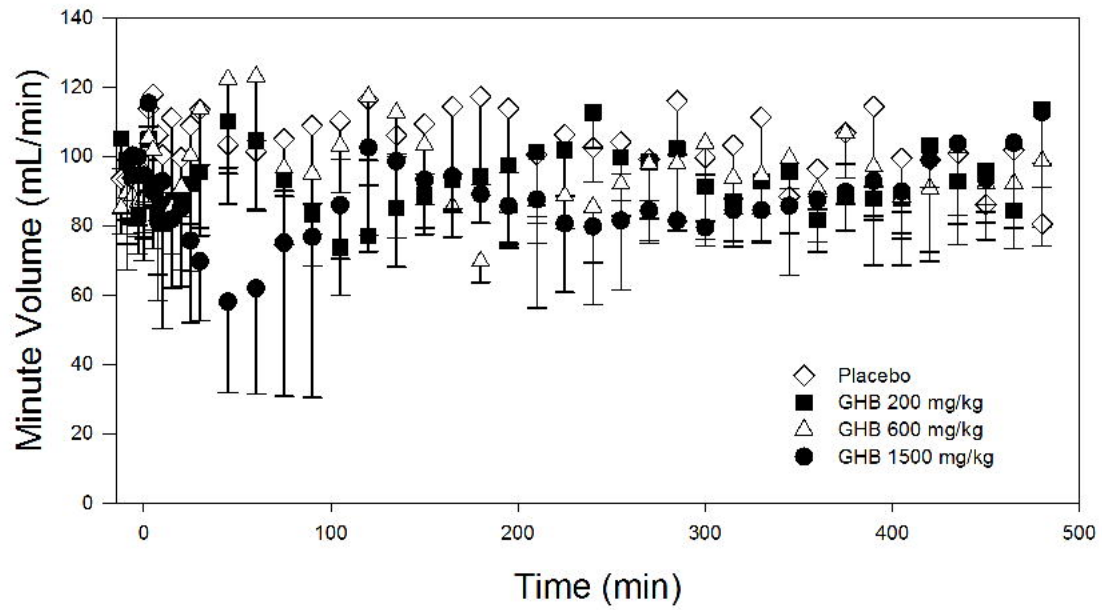

Minute Volume (mL/min)
Time (min)
Placebo
GHB 200 mg/kg
GHB 600 mg/kg
GHB 1500 mg/kg

Fig. 2

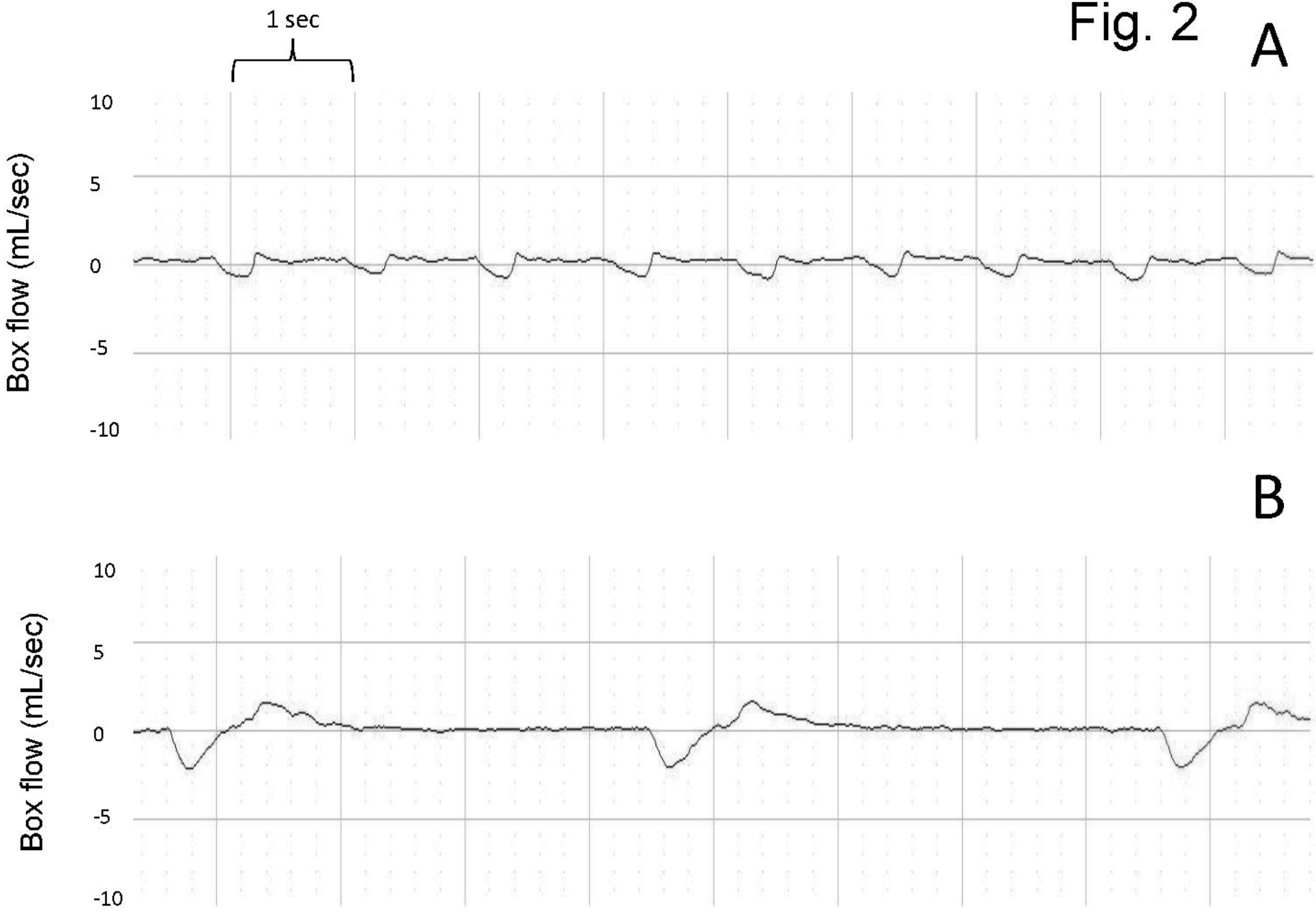
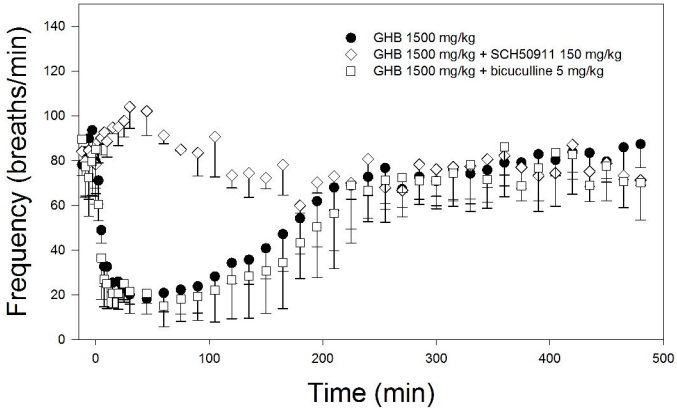


Fig. 3


GHB 1500 mg/kg
GHB 1500 mg/kg + SCH50911 150 mg/kg
GHB 1500 mg/kg + bicuculline 5 mg/kg
Frequency (breaths/min)
Time (min)
0
20
40
60
80
100
120
140
0
100
200
300
400
500

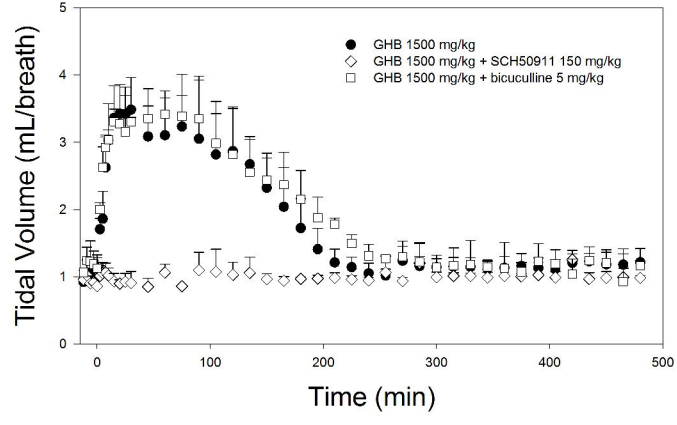

GHB 1500 mg/kg
GHB 1500 mg/kg + SCH50911 150 mg/kg
GHB 1500 mg/kg + bicuculline 5 mg/kg
Tidal Volume (mL/breath)
Time (min)
0
1
2
3
4
5
0
100
200
300
400
500

Fig. 4

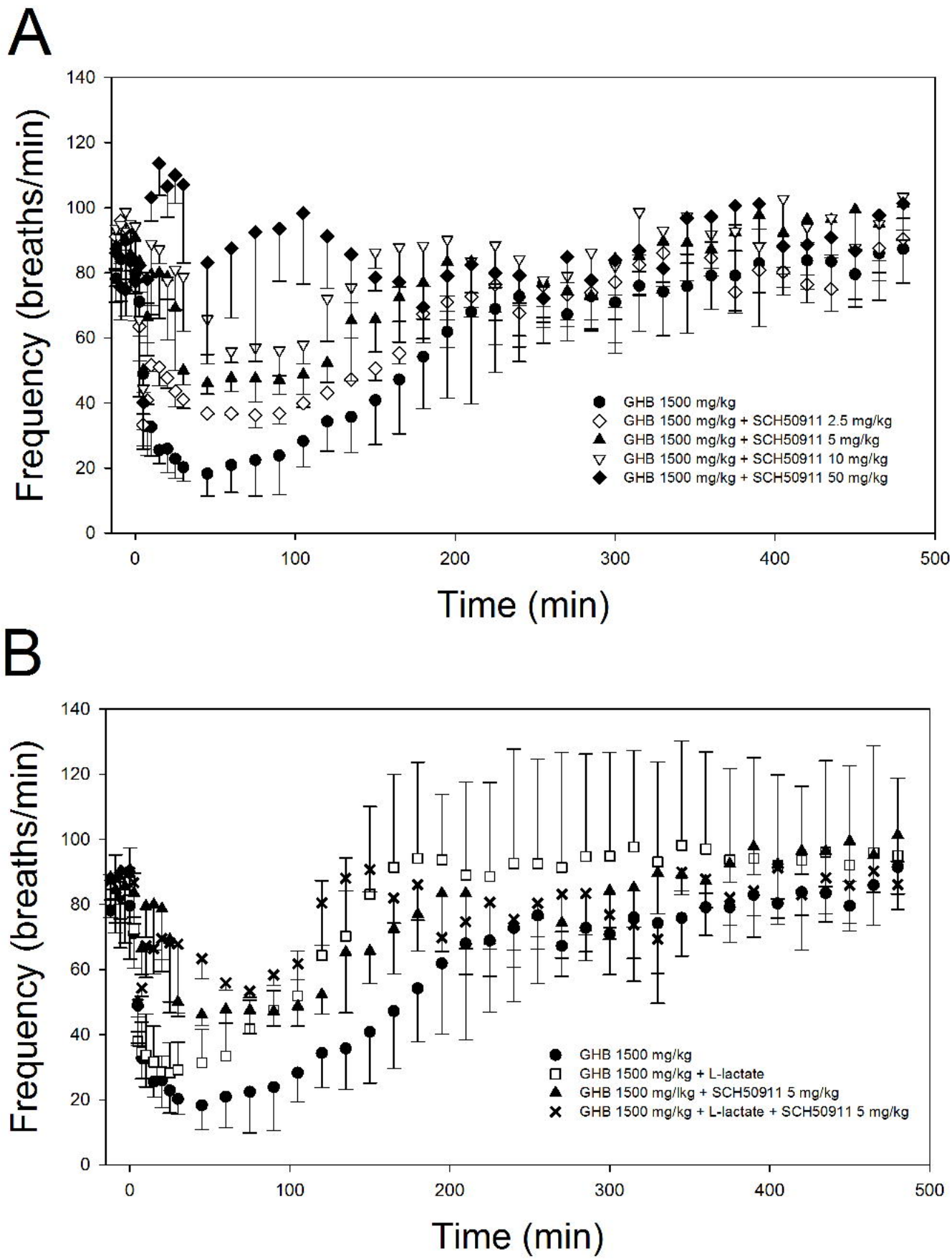

A
Frequency (breaths/min)
Time (min)
GHB 1500 mg/kg
GHB 1500 mg/kg + SCH50911 2.5 mg/kg
GHB 1500 mg/kg + SCH50911 5 mg/kg
GHB 1500 mg/kg + SCH50911 10 mg/kg
GHB 1500 mg/kg + SCH50911 50 mg/kg
B
Frequency (breaths/min)
Time (min)
GHB 1500 mg/kg
GHB 1500 mg/kg + L-lactate
GHB 1500 mg/lkg + SCH50911 5 mg/kg
GHB 1500 mg/kg + L-lactate + SCH50911 5 mg/kg

Fig. 5

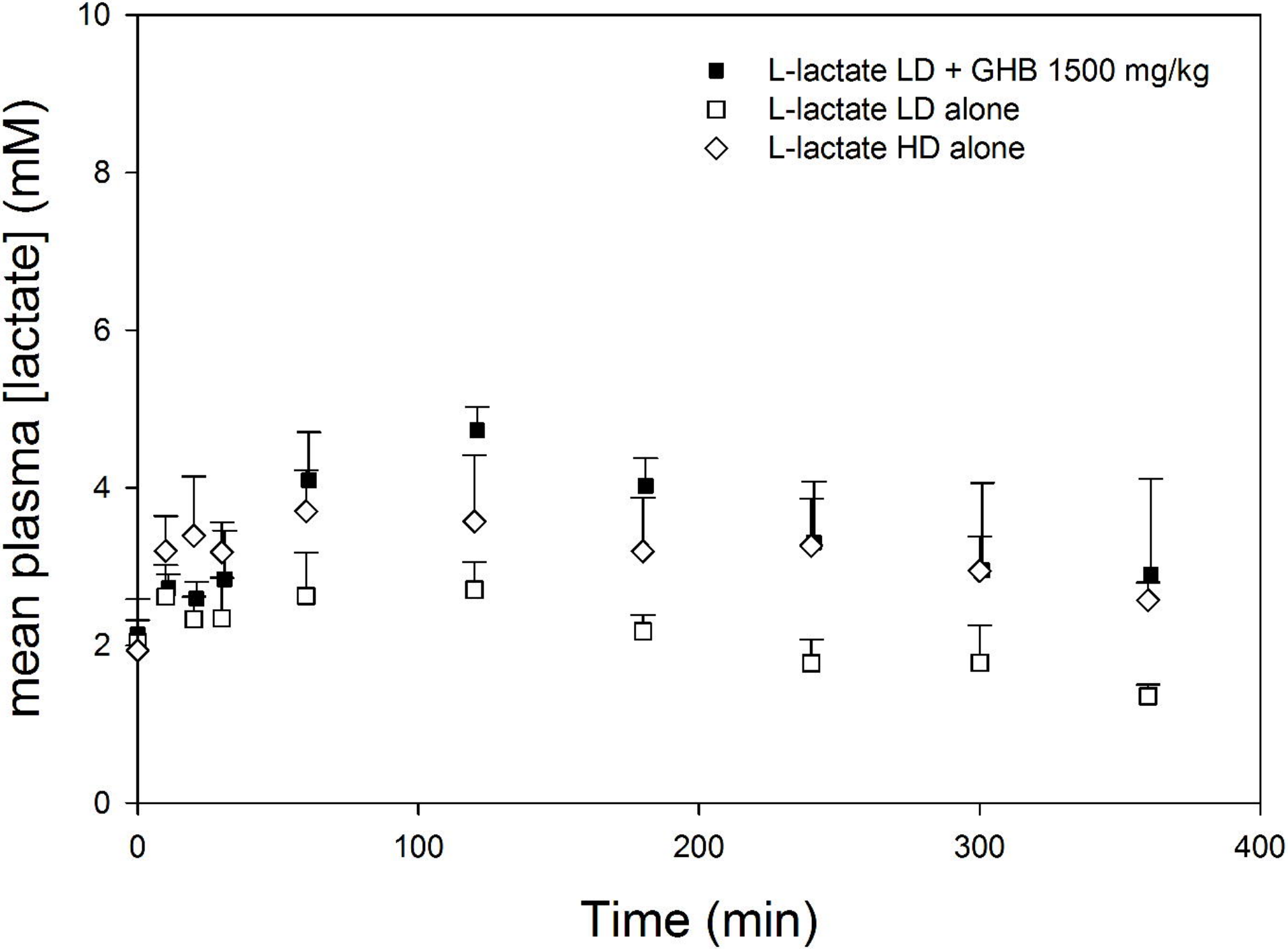
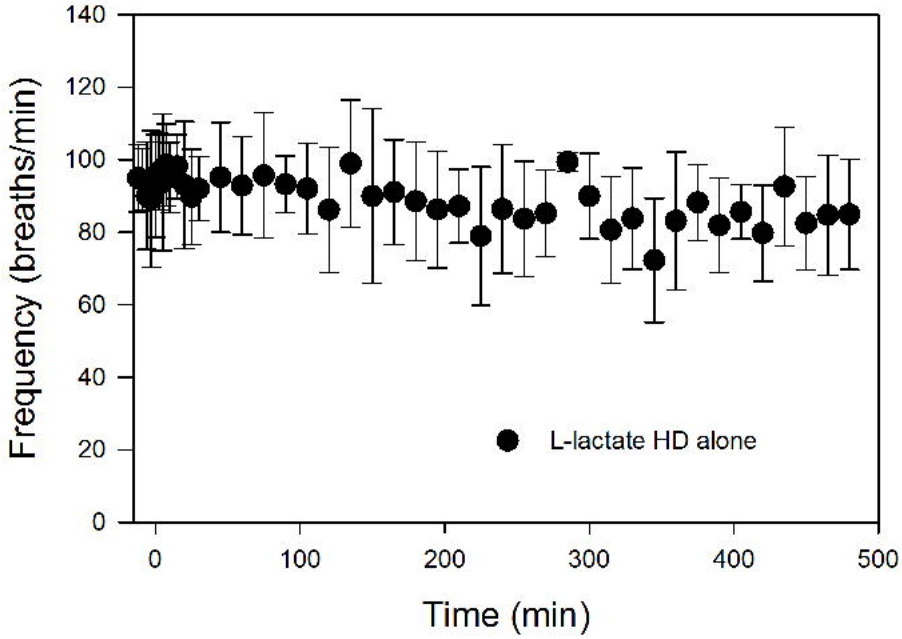
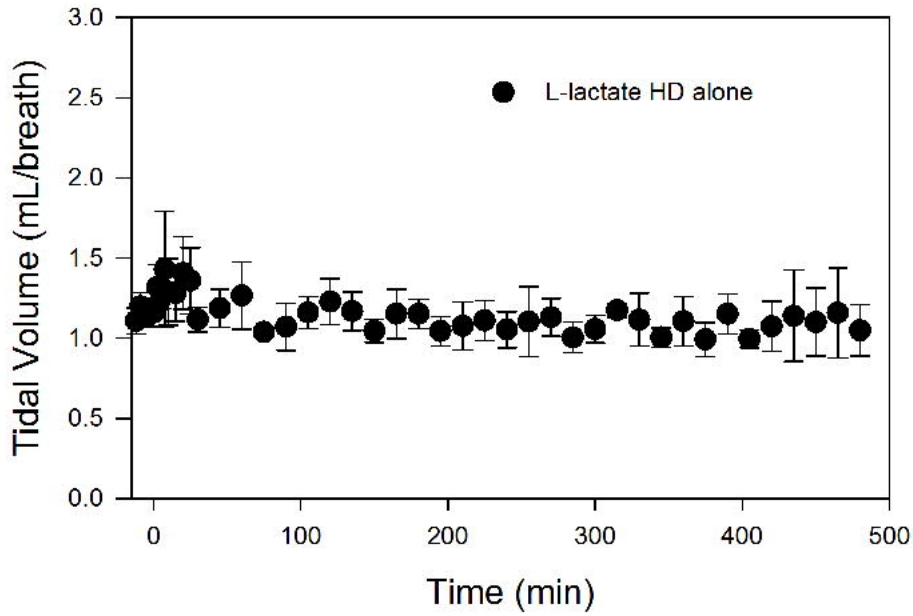
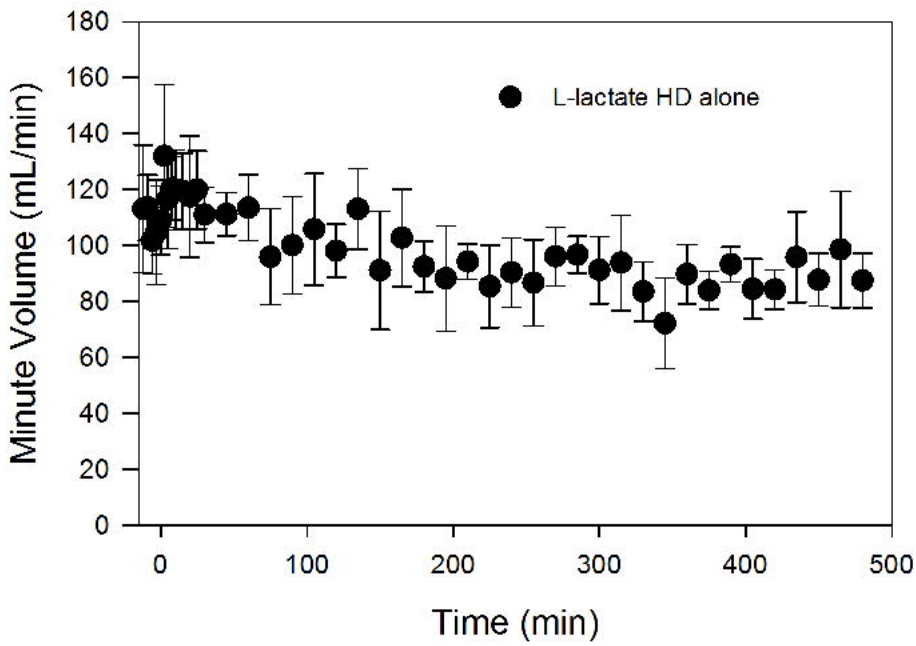

10
8
6
4
2
0
mean plasma [lactate] (mM)
0
100
200
300
400
Time (min)
L-lactate LD + GHB 1500 mg/kg
L-lactate LD alone
L-lactate HD alone

Fig. 6


Frequency (breaths/min)
140
120
100
80
60
40
20
0
0
100
200
300
400
500
Time (min)
L-lactate HD alone


Tidal Volume (mL/breath)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
500
Time (min)
L-lactate HD alone


Minute Volume (mL/min)
180
160
140
120
100
80
60
40
20
0
0
100
200
300
400
500
Time (min)
L-lactate HD alone