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Acute tolerance to rate-decreasing effects of single doses of ethanol

Brett C. Ginsburg, Ph.D.[1], **Gerardo Martinez, B.S.**[1], **Gregory Friesenhahn, B.S.**[1], **Martin Javors, Ph.D.**[1,2], and **R.J. Lamb, Ph.D.**[1,2]

[1]Department of Psychiatry, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900

[2]Department of Pharmacology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900

Abstract

Acute tolerance occurs when behavioral impairment is greater at a given blood ethanol concentration (BAC) on the ascending versus descending limb of the BAC-time curve following administration of a single dose of ethanol, however studies utilizing learned behaviors have not been widely reported. We assessed acute tolerance to single doses of ethanol in five Lewis rats responding under a fixed-ratio (FR8) schedule of food presentation. Response rates for food during 1-min components (ending 2, 4, 11, 18, 33, and 57 min after ethanol administration) were determined, and BAC was measured immediately after each component using a rat breathalyzer. Ethanol (0.4, 0.6, 0.8, and 1.2 g/kg, i.p.) produced dose-related decreases in responding for food that tended to recover over time for all but the highest dose tested. Similarly, dose-related increases in BAC were also observed. Using either an analysis that expressed impairment per unit BAC on the ascending limb versus the descending limb (by assessing the area under the curve (AUC) for behavior and BAC on each limb), the slope of the function that relates the behavioral effect to BAC (each expressed as percent maximum effect), or a variant of the Mellanby method (hysteresis), acute tolerance was observed following a dose of 0.4 g/kg ethanol. Though behavior appered to recover on the descending limb following higher doses (especially 0.6 and 0.8 g/kg), acute tolerance to these doses was not present.

Keywords

alcohol; rebreathed air; Mellanby effect; operant

Introduction

Acute tolerance occurs when behavioral impairment is greater at a given blood ethanol concentration (BAC) on the ascending versus descending limb of the BAC-time curve following administration of a single dose of ethanol. Sir Edward Mellanby (1919) published the first study demonstrating acute tolerance to ethanol. In that study, ataxia in dogs produced by ethanol was greater at a given BAC on the ascending versus descending limb of

Corresponding author: B.C. Ginsburg, ginsburg@uthscsa.edu, Department of Psychiatry, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr. San Antonio, TX 78229-3900, Phone: 210-567-0871, FAX: 210-567-5381.

the BAC-time curve (Mellanby, 1919). The phenomenon of acute tolerance may help clarify processes involved in alcohol dependence as well as in learning and memory (Kalant, 1998). Acute tolerance to ethanol has been reported in humans and animals, using measures such as subjective ratings (or ethanol discrimination in animals), ataxia, loss of the righting reflex, and impairment of psychomotor function.

In humans, Radlow and Hurst (1985) demonstrated that acute tolerance to subjective ratings of ethanol effects begins immediately after ethanol administration, and progresses as a linear function of time, independent of dose. Acute tolerance to ethanol also develops in humans when ataxia is the measure (Wang et al., 1993). In another study, acute tolerance developed to the slowing of reaction time due to ethanol, but not to the increased failure to inhibit a response due to ethanol in humans performing a go/no-go task (Fillmore et al, 2005). Thus, some behavioral tasks appear more susceptible to acute tolerance than others. In these studies, BAC was measured with a breathalyzer device. Measures of breath ethanol have been shown to more accurately reflect brain ethanol concentration when compared to ethanol concentration in venous blood, particularly during the ascending phase (*see* Pohorecky and Brick, 1982).

In animal studies, several laboratories have investigated acute tolerance to ethanol using measures that do not require training. The first report of acute tolerance was by Mellanby (1919) who showed that motor impairment in dogs was greater on the acscending portion of the BAC-time curve compared with the descending portion. In rats, the BAC upon recovery of the righting reflex following an initial administration of ethanol is lower than the BAC upon recovery following a subsequent administration of ethanol (Silveri and Spear, 1998). Similarly, in another study, acute tolerance to ethanol effects on rearing behavior in an open field was reported (Hiltunen and Jarbe, 1990). Acute tolerance to the hypothermic effects of ethanol has also been demonstrated in mice, and the degree of tolerance may be strain- or subject dependent (Radcliffe et al., 2006; San-Marina et al., 1989). These studies demonstrate that acute tolerance develops to ataxic and hypothermic effects of ethanol.

Few studies of acute tolerance have been conducted utilizing learned behaviors. LeBlanc et al., (1975) trained rats to stay on a moving belt. Subjects that stepped off of the belt received a mild electric shock. Impairment, measured as time off of the belt was regressed against brain ethanol concentrations immediately after the test. The regression line of impairment as a function of brain ethanol concentration showed a progressive rightward shift (toward higher effective brain concentrations) with increasing time after alcohol administration (LeBlanc et al., 1975). Others have extended these findings by demonstrating that practice during intoxication enhances the development of acute tolerance in this procedure (Lê and Kalant, 1992). Hiltunen and Jarbe (1990) demonstrated acute tolerance to ethanol in rats trained to discriminate ethanol from saline using a fixed-ratio schedule, but only for doses above the training dose. However, acute tolerance was present only for doses of ethanol at or above the training dose. Because drug discrimination requires repeated administration of the training dose, it is likely that chronic tolerance to ethanol had developed in these rats (*see* Hiltunen and Jarbe, 1992). Subsequently, the same authors showed that acute tolerance developed in rats trained to respond under a fixed-ratio schedule for food. Further, in rats treated chronically with ethanol, acute tolerance was only present at ethanol doses above the chronic dose (Hiltunen and Jarbe, 1992). In this study, different doses of ethanol were administered to separate groups of subjects to provide equal breath ethanol concentrations at 10-min (ascending limb) and 60-min (descending limb) following administration. Fixed ratio responding for food was assessed at each time point in separate groups of rats. Thus, full dose-effect curves on the ascending and descending portions of the BAC curve were not determined. Further, comparisons were between-subjects.

In the present study, acute tolerance to a single dose of ethanol was assessed in rats performing under a fixed-ratio schedule of responding for food. Results of analyses with the AUC measure described by Martin and Moss (1993), the slope-function method described by Radlow (1994), and hysteresis plots are reported. Rats were trained to respond for food during six 1-min components ending at 2, 4, 11, 18, 33, and 57 minutes after ethanol (or vehicle) administration. BAC was determined with a rat breathalyzer we have previously described (Javors et al., 2005) immediately following each component. This procedure provides a non-invasive means to determine acute tolerance to impaired operant responding following various ethanol doses across time, as well as allowing within-subjects analysis of tolerance.

Materials and Methods

Subjects

Five male Lewis rats (Harlan, Inc, Indianapolis, IN) were individually housed in a colony room with an average temperature of 20°C and a 14/10-h light/dark cycle (experiments were performed during the light cycle). Rats initially weighed between 200–250g, and were allowed at least 2 weeks to habituate to vivarium routines. During this period, weights were allowed to increase to 300–350g. Food (Purina Rat Chow, Purina, St. Louis, MO) was then restricted to approximately 10–15g/day (fed after each daily behavioral session) to maintain rats at body weights of 300–325g. Rats had *ad libidum* access to water in their home cages.

Apparatus

Behavior—Five commercially available operant conditioning chambers (MedAssociates, Georgia, VT) were equipped with two response levers, two lever lights (one above each lever), a house light, and a pellet magazine between the response levers. Each chamber was enclosed in a sound and light attenuating box and vented with an exhaust fan (Med Associates, Georgia, VT). White noise generators provided an experimenter-controlled sound stimulus via a small speaker in each chamber. Contingent light and food pellet presentations were coordinated and lever responses were recorded with commercially available software (MedPC, MedAssociates, Georgia, VT).

BAC—Breath ethanol concentration was measured using a device we have previously described (Javors et al., 2005). This device was modified from that described by others (Pohorecky and Brick, 1982; Hiltunen and Jarbe, 1990). Briefly, the rat breathalyzer consisted of a polycarbonate body chamber (22 cm long, 6 cm i.d.) to secure the rat and a head chamber (4 cm long, 5 cm i.d.). The rat's head protrudes from the body chamber. The head of the rat is placed into this head chamber during the 30-sec breath sampling. A 30 cm long stainless steel tube (1/16" o.d. 0.01" i.d.) ran from the head chamber through a 1-ml sample loop and then to the injector port on a Thermo Finnigan Trace gas chromatograph (GC) system equipped with a flame ionization detector (FID). A Gilson Minipuls 2 calibrated peristaltic pump drew the breath sample from the head chamber and filled the sample loop.

Procedure

Behavior—Initially, rats were trained to respond for food in the presence of an illuminated stimulus light above the active lever and the presence of white noise. During daily sessions of a single one-hour component, each response resulted in delivery of two 45 mg food pellets (rat chow flavor 45 mg, Research Diets, Inc., New Brunswick, NJ), and illumination of the house light for 3-sec. During this 3-sec timeout, the stimulus light above the lever and the white noise were extinguished, and responses had no programmed consequences. Once rats earned 200 pellets (100 responses) over two consecutive days, the response requirement

was gradually increased to eight (FR8). After rats reliably responded under the FR8 over the single component session, the single hour-long session was split into two 20-min sessions, separated by a 20-min timeout period in which no stimuli were present, and responses had no programmed consequence. Eventually, these two components were separated into a total of six components, each lasting 5-min and separated by a 5-min timeout. Gradually, the length of each of the six components was reduced to 1-min. Starting at time zero, the 1-min FR8 components ended at 2, 4, 11, 18, 33, and 57-min. Rats were allowed to continue under this schedule until each attained stable behavior, defined as a standard deviation < 20% of the mean number of responses in each component across five consecutive days.

BAC—Once behavior had stabilized on the final schedule, rats were introduced to the breathalyzer. Habituation continued daily as described below until responding again stabilized, when experiments commenced. Following each component, each rat was removed from the operant chamber and placed in the body chamber of the breathalyzer apparatus. The rat was then taken to an adjacent room with the head chamber and chromatography equipment. The rat's head was positioned in the head chamber for 30-sec. During this equilibration period, the end of the sampling tube was open to the head chamber and the peristaltic pump was not activated. A sample of the rebreathed air was drawn from the head chamber for 6 sec at a flow rate of 22 ml/min. The sample was injected onto the GC where ethanol was quantified by comparison with an ethanol calibration curve, generated daily prior to each experiment. Each BAC measurement took just under 1-min.

Ethanol administration—Ethanol was dissolved in 0.9% saline at a concentration of 10% (w/v). Varying amounts of ethanol were administered (i.p.) by injecting the appropriate volume of the ethanol solution. Vehicle, as well as doses of 0.4, 0.6, 0.8, and 1.2 g/kg were tested. Dose order for each rat was randomized using a Latin square design. Experiments were conducted once per week to permit recovery between ethanol exposures. Subsequent analysis revealed no effect of the order of dosing on either breath ethanol or behavioral measures. Thus, no effect of dose-order was present for response rate, though a main effect of dose was present ($F[4, 7]=10.5$, $p<0.05$). The interaction between dose and order was not significant. Similarly, there was no effect of order on BrAC , though there was a main effect of dose ($F[4, 7] = 32.6$, $p<0.05$). Again, no interaction between dose and order was present for BrAC.

Analysis

Behavior—Total number of responses per 1-min component was recorded for each component in each subject following each ethanol dose. Response rate was calculated by dividing the total number of responses per component by the amount of time in which the lever was active during the 1-min component. For subsequent analyses of acute tolerance, response rates for each subject were normalized by expressing the rate following each dose of ethanol during each component as a percentage of the rate following saline during the corresponding component.

BAC—BAC, expressed in mM, was calculated using the conversion factor we have previously reported which relates ethanol measured in rebreathed air with arterial blood ethanol concentration (Javors et al., 2005). BAC was determined in each subject immediately after each behavioral component following each ethanol dose or saline vehicle.

Acute tolerance—We used several different techniques to evaluate the appearance of acute tolerance, including the AUC method and the modified Radlow method as described by Martin and Moss (1993), as well as hysteresis curves. Finally, we compared the A50 for BAC on behavior on the ascending and descending portions of the curve in an attempt to use

a pharmacologically validated method of assessing acute tolerance. Each of these methods is described below.

AUC method—In this procedure, the ratio of the (AUC for intoxication) / (AUC for BAC) over the ascending and descending limbs of the curve are compared. The equations used are shown below:

$$\text{ASC}=\text{Ascending}\ (\text{AUC}_{\text{behavior}}/\text{AUC}_{\text{BAC}}) \quad (1)$$

$$\text{DEC}=\text{Descending}\ (\text{AUC}_{\text{behavior}}/\text{AUC}_{\text{BAC}}) \quad (2)$$

$$\text{C}=\text{DEC}/\text{ASC} \quad (3)$$

For each subject, the AUC for the ascending portion of the BAC-time curve was integrated from the first time point (1-min) to the time point where BAC was maximal for the group at each ethanol dose. The AUC for the behavior-time curve was similarly integrated from the first time point to the time point of maximal group BAC for each dose. Likewise, the descending AUC for each measure (BAC and behavior) was determined from the time point of maximal group BAC for each dose to the last time point (57-min). Time was LOG transformed to normalize the distribution. While *larger* AUC for BAC indicate *greater* ethanol concentrations over time, *smaller* behavior AUC indicate *greater* impairment over time. The value of the ascending and descending limb ratios (ASC and DEC) were calculated by dividing behavior-AUC / BAC-AUC for each segment. This provides a measure of impairment per unit BAC for each segment of the curve. Thus, relatively smaller values of ASC or DEC represent relatively greater impairment per unit BAC. So, if DEC>ASC, impairment per unit BAC is relatively less on the descending limb of the curve. Acute tolerance was considered to have occurred if the ratio for the ascending limb was greater than the ratio for the descending limb (from Eq. 3: C > 1). That is, the impairment per unit BAC is lower for the descending versus ascending limb.

Slope function—Radlow (1994) proposed that acute tolerance begins upon ethanol administration, proceeds as an increasing function of time, and can be measured by relating BAC and behavioral effects across time. Martin and Moss (1993) adapted this technique by converting BAC and behavior to percent maximum effect following a single dose of ethanol. In the present study, behavioral percent maximum effect for each subject at each time point (% MPE(t)) was calculated using equation 4 for each dose and time point.

$$\%\ \text{MPE}(t)=100-\text{RR}(t) \quad (4)$$

Where RR(t) = Response rate (normalized to time matched saline control) at a given dose and time point in a subject.

The difference between percent maximum BAC and percent maximum behavioral effect is determined in each subject at each time point. This function is then plotted over time. The rate (slope) of this function over time provides a measure of acute tolerance. Slopes were calculated across time points that included the last point where response rate was significantly different from control, and the next time point. Later time points (that were no different from control) were considered to reflect complete recovery of behavior, when no

further acute tolerance is possible. For 0.4 g/kg, points collected after 11-min were excluded. For each of the other doses, all points are included. A positive slope is indicative of the development of acute tolerance, as the difference between BAC and behavior grows over time (due a faster rate of behavioral recovery compared with elimination of ethanol).

Hysteresis plot—Hysteresis describes the behavior of a system in which the observed effect (in this case behavioral impairment) of an external force (in this case BAC) is not in direct concordance. By plotting the behavioral effect against BAC for each dose across time, the relationship between these measures can be visualized. For each dose, paired t-tests were performed to compare behavior at each time point on the ascending limb with each time point on the descending limb. Similar analyses were performed on BAC values following each ethanol dose. The resulting p-values were then corrected for multiple comparisons with the Benjamani-Hochberg method (Benjamini and Hochberg, 1995).

A50 method—The A50 is the dose of ethanol which results in a 50 percent reduction in responding for ethanol by the group. By comparing A50 values for time points on the ascending and descending limbs of the BAC – time curve, changes in the potency of ethanol can be established. If lower doses of ethanol reduce behavior by 50% at early time points compared with later time points, this would indicate the development of acute tolerance.

The method described by Tallarida and Murray (1987) of calculating the A50 was used to determine A50 for BAC on behavior at each time point. Briefly, points with behavior greater than 80% or less than 20% of the control rate were excluded to better represent the linear portion of the BAC-behavior curve. For each time interval, BAC was expressed as LOG(BAC), and a linear regression was performed across all remaining points. The sum of squared differences between each BAC value and the average BAC was calculated, and the sum of squared differences between each behavioral effect (expressed as percent control) and the behavioral effect predicted by the overall regression was calculated. These values were then used to calculate the A50 and corresponding confidence limits at each time interval

Statistics and data management—All regressions, analyses, and graphs were produced using the R statistics package (R Development Core Team, 2005). AUC was derived by using the *integrate.xy* function of the **sfsmisc** package for R. Boundaries for integration of the ascending limb were set from the first time point to the time point of maximum BAC for the group at each dose. Boundaries for the descending limb were set from the point of maximum BAC to the last time point. A smoothing spline was not used, and the time values were log transformed to normalize their distribution. Response rates were expressed as a percentage of each subject's control rate at each time point.

Two-tailed t-tests were performed for the AUC measure results. The p-values derived from the t-tests were corrected for multiple comparisons with the Benjamini-Hochberg method. This method (which is a variant of the Hochberg step-down method) controls the false discovery rate, while maintaining high power.

The slope of the function relating BAC and behavior was compared with zero to establish whether the slope was positive. To do this, a linear regression was performed on points up to and including the last time point where a significant behavioral impairment was detected, as well as the next time point. The standard error of the resulting slope was multiplied by the appropriate Student's t distribution value at 95% confidence and added to and subtracted from the regressed slope. If the 95% confidence limits of the slope did not include zero, the slope was considered to be positive.

Results

The time course and magnitude of the effects of ethanol on behavior (Fig. 1A) and BAC (Fig. 1B) were similar to those previously reported (Hiltunen et al., 1989). At doses of 0.4, 0.6 and 0.8 g/kg, responding for food was initially impaired, but appeared to recover by 11–18 min after ethanol administration. A higher dose of ethanol (1.2 g/kg) produced impairment that persisted throughout the 57-min experimental period. Following doses of 0.4, 0.6, or 1.2 g/kg ethanol, BAC reached maximal levels 4-min after administration (Fig. 1B). Maximal BAC following 0.8 g/kg was achieved 11-min after administration. BAC remained elevated following each dose throughout the 57-min experimental period, except for 0.4 g/kg which had returned to control levels 57-min after administration.

AUC measure

The results of this analysis are summarized in Table 1. Overall, the AUC for BAC was lower following 0.4 g/kg as compared to 1.2 g/kg; indicating increasing BAC. The AUC for behavior decreased with dose (indicating increasing impairment), as expected. As described in the Methods section, if the value of C from Eq. 3 is greater than 1 (that is, if descending AUC ratio > ascending AUC ratio), acute tolerance is present. Mean values of C were greater than 1 for 0.4 and 0.6 g/kg ethanol. As shown in Table 1, t-tests were performed in which C for all subjects following each ethanol dose were compared with 1 and corrected for multiple comparisons with the Benjamani-Hochberg method. Based on group results, the AUC measure was significantly greater than 1 after 0.4 g/kg ethanol ($p<0.05$).

When individual subjects are considered, C was greater than 1 in all five subjects after the 0.4 g/kg dose and in four of five subjects following the 0.6 g/kg ethanol dose. Acute tolerance was observed in only two subjects following the 0.8 g/kg ethanol dose; however one subject exhibited no behavior on the ascending limb of the BAC curve, resulting in an indeterminable value of C. Another subject did not recover behavioral function on the descending limb, resulting in C = 0.05. After the 1.2 g/kg dose of ethanol, two subjects exhibited acute tolerance, while one subject exhibited no behavior on the ascending limb resulting in an indeterminable value of C. Thus, considered across individual subjects, and as a group, acute tolerance appeared most pronounced after 0.4 g/kg ethanol. Although results following 0.6 g/kg ethanol failed to reach significance, some evidence of acute tolerance was evident at the individual subject level. No consistent evidence of acute tolerance was present following doses of 0.8 or 1.2 g/kg ethanol.

Slope function measure

In a method first proposed by Radlow and Hurst (1985) and modified by Martin and Moss (1993), a positive slope of the difference between behavioral effect and BAC (expressed as a percent of maximum effect) over time indicates acute tolerance. The results of this analysis are presented in Table 2 and Fig. 2. Only the slope function following 0.4 g/kg ethanol was significantly greater than zero, and represented the best fit among all of the doses tested ($r=0.75$). Positive slopes were obtained for 0.6, 0.8, and 1.2 g/kg, however these slopes were not significantly different from zero.

As shown in Fig. 2, the slope function (solid circles) represents the difference between the normalized breath ethanol curve (triangles) and the normalized effects of ethanol on behavior (squares) at each time point. Recovery of behavior while BAC rises or remains constant results in a positive slope (most readily seen for the 0.4 g/kg dose over the first 11 minutes).

Hysteresis plots

Group hysteresis plots for 0.4, 0.6, and 0.8 g/kg doses are presented in Fig. 3. AUC measure and slope function analyses indicated that acute tolerance was most likely at these doses. In these plots, the behavioral effect (as percent control) is plotted as a function of BAC at the corresponding time point for the group data. Each point is labeled to show the time point it represents. Vertical bars represent S.E.M. of response rate and horizontal bars represent the S.E.M for BAC at each time point following the appropriate ethanol dose. Vertical movement with increasing time suggests acute tolerance, as response rate is recovering while BAC remains the same in this condition. Following a dose of 0.4 g/kg ethanol (Fig. 3A), response rate recovers with little change in the BAC for the 4-min and 18-min post injection points. Indeed, a paired, t-test corrected for multiple comparisons revealed that response rate was significantly lower at 4-min versus 18-min ($t[4]=6.85$, $p<0.05$). Yet, BAC was not significantly different at the same two time points. Following a dose of 0.6 g/kg ethanol (Fig. 3B), comparison of response rate at 4-min with rates at time points of 33-min and 57-min was significant ($t[4] = 2.91$ & 4.21, $p<0.05$, respectively), though corresponding BAC were not different. Similar comparisons following 0.8 g/kg ethanol (Fig. 3C), did not reveal any significant differences between response rates on the ascending versus descending limb. Thus, these results are generally in agreement with the AUC measure method and the slope function method.

A50 calculations

The results of these analyses are summarized in Table 3. The A50 at 4-min was much lower than the A50 at 33-min; in fact, the A50 at 4-min fell below the lower 95% confidence limit of the A50 at 33-min, though the converse was not true, and other A50 values did not differ. However, it is difficult to make strong conclusions about these results. The limited number of data points and the lack of a convincing relationship between BAC and behavior, especially at early time points were problematic. Indeed, a positive relationship was present between BAC and behavior (higher BAC resulted in less behavioral disruption) at the 2-min time point. Such a relationship makes interpreting the A50 calculation at this time point difficult at best.

Discussion

Our results demonstrate that acute tolerance to the effects of ethanol can be assessed using fixed-ratio responding for food coupled with a rat breathalyzer. This method is non-invasive, and can be used to assess acute tolerance to impairing (but not incapacitating) doses of ethanol. Further, the use of the AUC measure method appears to be a reasonable means of confirming the presence of acute tolerance, without sacrificing data points. Analysis of the slope function method and hysteresis curves (providing a method similar to the classic Mellanby method) provided complementary results. While consistent, the A50 method of analysis was less convincing. Our procedure allows within-subject, single dose examination of the phenomenon of acute tolerance. Although some limitations are noted, this study could form the basis of further studies on the impact of practice during intoxication, route of ethanol administration, and perhaps even specific brain region involvement in the development of acute tolerance.

The AUC measure method provided evidence that acute tolerance occurred following a dose of 0.4 g/kg ethanol. Acute tolerance was not confirmed with the AUC measure method following doses of 0.6, 0.8 or 1.2 g/kg ethanol. For the highest dose tested (1.2 g/kg), this was clearly due to a floor effect where responding was almost completely eliminated throughout the duration of the experiment.

The slope function measure also revealed acute tolerance following 0.4 g/kg ethanol. The slopes following 0.8, 0.6 and 1.2 g/kg ethanol, while also positive, were not significantly greater than zero. Thus, the slope function result is consistent with the AUC measure result.

Examination of hysteresis plots reveals acute tolerance at specific time points following the lowest ethanol doses examined. Specifically, time points following doses of 0.4 and 0.6 g/kg (but not 0.8 or 1.2 g/kg) were identified at which behavior had significantly recovered while BAC remained unchanged. This is similar to results of the AUC measure and slope function methods described above. In a previous study, Martin and Moss (1993) found that the classic Mellanby method of assessing acute tolerance was well correlated with the AUC measure method, but not the slope function method. Our results are in agreement with their conclusions, as our analysis of the hysteresis function is essentially a variant of the classic Mellanby method.

The present study is an extension of earlier work, especially studies performed by Hiltunen and Jarbe (1990, 1992). In those studies, the authors used operant techniques and a breathalyzer apparatus similar to the one used in the present study. Hiltunen and Jarbe (1992) assessed acute tolerance to ethanol-induced reductions in fixed-ratio responding for food by determining effects of two different doses at two different time points in two different groups of rats. The selected dose-time pairs resulted in equal BAC (one on the ascending and one on the descending limb). The classic Mellanby method was then used to establish that acute tolerance occurred in ethanol-naïve rats (Hiltunen and Jarbe, 1992). Acute tolerance was also seen in rats chronically treated with ethanol, but only following doses higher than the chronic dose (Hiltunen and Jarbe, 1992). This finding was similar to an earlier finding utilizing drug discrimination as the behavioral measure (Hiltunen and Jarbe, 1990). In the present study, acute tolerance to the rate-decreasing effects of a single dose of ethanol in a single subject within an experimental session was demonstrated.

Demonstration of acute tolerance following a single dose of 0.4 g/kg is similar to the findings of others when the measure is a learned behavior. In one study by Fillmore et al., (2005), humans received 0.65 g/kg ethanol and acute tolerance was observed on initiation, but not suppression of a response in a go/no-go task (Fillmore et al., 2005). The present demonstration of acute tolerance to a single dose of 0.4 g/kg represents the lowest dose reported to result in acute tolerance in rats to a single dose of ethanol in the literature. In other studies (Pomomarev and Crabbe, 2002) acute tolerance to the hypnotic effects of a higher dose of ethanol (3 g/kg) were observed. In the present study, the highest dose tested (1.2 g/kg) eliminated fixed ratio responding throughout the session, making detection of acute tolerance impossible. Intermediate doses did not produce acute tolerance. This could reflect different sensitivity of different behaviors to acute tolerance to ethanol.

Indeed, the behavior we measured represents a limitation of our study. Fixed ratio responding for food is very sensitive to disruption by ethanol (compared with other behaviors such as loss of the righting reflex). In addition, appetitive effects of ethanol have been documented (Caton et al., 2007). Ethanol appears to increase appetite immediately after ethanol ingestion, and stimulates food intake early in a meal. Because impairment in our study was greatest in the first few minutes of each session, such appetite stimulating effects might oppose the impairment of responding for food at these early time points. Thus, appetitive stimulation by ethanol could reduce our ability to see acute tolerance (by reducing the maximum impairment seen on the ascending limb of the BAC curve).

Another limitation of this study is the relatively low degree of behavior generated by rats responding on a lever during one-minute periods. This limits the resolution available to assess impairment. Thus, small changes in the number of responses completed within the

component result in large changes in response rate. Such restrictive behavioral parameters could exaggerate the effects of ethanol. Reducing the post-reinforcement timeout, increasing the component length, or both, could provide less restrictive behavioral parameters by increasing the amount of time that rats can respond for food in each component. Additionally, the variance in the control condition is relatively high, which reduces the resolution with which changes can be assessed. Use of an operant that is less demanding on time and effort could lessen this limitation. One potential method would be the use of head entry detectors which might allow generation of higher rates of responding compared with levers under the same experimental conditions.

A related limitation is the necessity of repeated handling of rats during each behavioral session in order to obtain breath samples. We took great care to habituate subjects to the procedure, and only proceeded with experiments once rats response rate over time was consistent over time during sessions that followed an injection and that contained repeated breath measurements. This requirement of extensive training could limit the feasibility of applying this procedure in studies in which long training periods are not practical.

One assumption that is critical to the interpretation of these data is that breath ethanol levels reflect brain ethanol levels. Fein and Meyerhoff (2000) demonstrated that in humans, breath ethanol measurements are well-correlated with brain ethanol levels measured with magnetic resonance imaging. In rats, the method used in the present study to measure breath ethanol levels is well-correlated with trunk venous blood on both the ascending and descending limbs of the ethanol-time curve (Javors et al., 2005). Thus, while measuring ethanol levels in tail blood tends to underestimate brain ethanol levels on the ascending limb (Nurmi et al., 1994), breath ethanol levels appear to provide an accurate estimation of changes in brain ethanol content. Despite the apparent feasibility of the AUC measure method, and the possible utility of the slope function method, each of these methods assumes a linear relationship between behavioral impairment and BAC. This assumption may not be valid across the doses tested. Indeed, Martin and Moss (1993) demonstrate that the slope function method, which relies on a linear relationship between behavioral effects and BAC does not describe this relationship on the ascending limb of the ethanol dose curve. Thus, it appears that the relationship between behavioral effects and BAC is non-linear on the ascending portion of the curve. This is also a limitation of the AUC measure method, which also depends on a linear relationship between behavior and BAC over both the ascending and descending portions of the curve in order to compare areas. Thus, it is possible that neither method is well suited to describe acute tolerance, leaving the classic Mellanby method as the most theoretically sound technique.

An alternative method could be to establish the relationship between behavior and BAC at various time points. Then, the BAC that results in a 50% reduction in behavior can be calculated (BAC50) and compared between time points on the ascending and descending portions of the time-effect curve. This is akin to calculating the ED50 and comparing shifts in potency in pharmacological studies. Significantly higher BAC50s at later time points would imply acute tolerance, as the “potency” of BAC levels is reduced. Although our analysis using this technique were consistent with other results, but not completely convincing, this is probably due to the rapid rise in BAC following i.p. dosing limiting our ability to collect data on the ascending portion of the curve, and high variance in our behavioral measure. Future studies could improve on our methods by slowing the rate of the rise of BAC, perhaps by using intragastric dosing. Further, by lengthening the amount of time in which rats can work for food in each component, either by reducing the post-reinforcement timeout, by increasing the component length, or both, could allow for the application of this method.

The application of a within-subjects method of assessing acute tolerance provides several advantages. This method allows increased experimental power as well as the ability to design ABA experiments. Assessing acute tolerance in rare or expensive subjects such as genetically modified or aged animals is facilitated by a within-subjects design. This procedure also allows dose-response relationships to be determined. Acute tolerance to other complex behaviors such as those used to study learning and memory (similar to studies performed in humans by Filmore, 2005) could be examined using a similar procedure.

The degree of acute tolerance expressed by an individual may be related to that individual's vulnerability to alcohol abuse (Bierness and Vogel-Sprott, 1984). The basic mechanisms underlying the development of acute tolerance are not yet completely understood. However, evidence supports the notion that there is an interaction between the impairing effects of ethanol and reinforcement (Vogel-Sprott, 1997). In particular, acute tolerance appears to develop in behaviors that are reinforced, but not in behaviors that are not reinforced (Vogel-Sprott, 1997). In the animal literature, several demonstrations of acute tolerance have used reinforced behavior (LeBlanc et al., 1974; Hiltunen and Jarbe, 1990; Holloway et al., 1992). Some have also demonstrated acute tolerance to the loss of righting reflex (which is not explicitly reinforced); though acute tolerance does not occur in other open field behaviors (Ponomarev and Crabbe, 2002; Tampier and Quintanilla, 2003; Hiltunen and Jarbe, 1990). The differences may provide an interesting insight into the mechanism which allows acute tolerance to develop for some, but not other behaviors, and could provide important information relevant to alcohol impairment in both alcoholic and non-alcoholic individuals.

In summary, acute tolerance was apparent following a dose of 0.4 g/kg ethanol. Acute tolerance may have been present following a dose of 0.6 and 0.8 g/kg ethanol, but the results failed to reach significance with most analyses. Acute tolerance to the highest dose tested, 1.2 g/kg was not observed, likely due to a floor effect of ethanol on our behavioral measure. In conclusion, the present study has demonstrated that fixed-ratio responding for food coupled with a rat breathalyzer can be used to assess the development of acute tolerance to ethanol in a single subject following a single dose within an experimental session. This procedure is non-invasive, and allows determination of acute tolerance at lower doses than procedures designed to examine ethanol-induced ataxia or loss of the righting reflex. As shown by Fillmore (2005), acute tolerance may develop differentially, depending on the behavioral measure. The present procedure allows an examination of acute tolerance development to complex, learned behaviors. The AUC measure method appears to be a reasonable and effective method for assessing the presence of acute tolerance, although evaluation of the magnitude of the effect remains difficult. Further, the assumption of a linear relationship between BAC and behavioral impairment remains problematic. The slope function method appears to be a less robust method of determining the presence of acute tolerance, and considering the results of Martin and Moss (1993) is not recommended.

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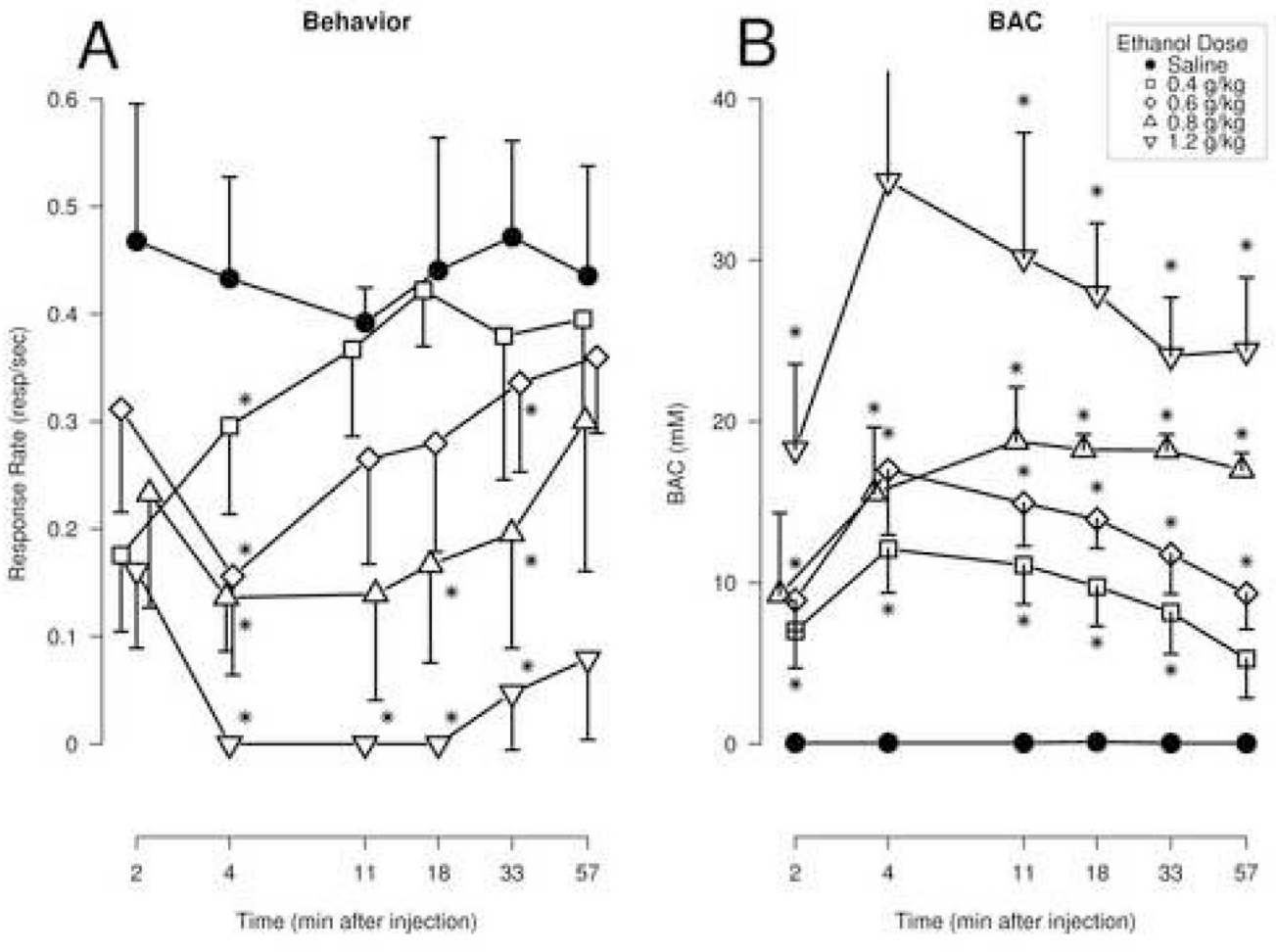

Figure. 1.

Effects of single doses of ethanol over time. Ethanol was administered at time zero. Each point represents the mean ± S.E.M. for 5 rats. Asterisks represent points significantly different from the time-matched saline control point as determined by paired t-tests corrected for multiple comparisons ($p<0.05$). A.) Behavioral impairment over time following several single doses of ethanol. Behavior was assessed over the 1-min period preceding the time indicated on the abscissa. B.) BAC over time following the same ethanol doses in the same subjects. BAC was determined in each subject immediately following the behavioral component ending at the time indicated on the abscissa. Points have been offset for clarity. NOTE: Although raw responses per minute are presented in this figure, data were normalized to each subject's time-matched saline control for all analyses (*see* Methods section)

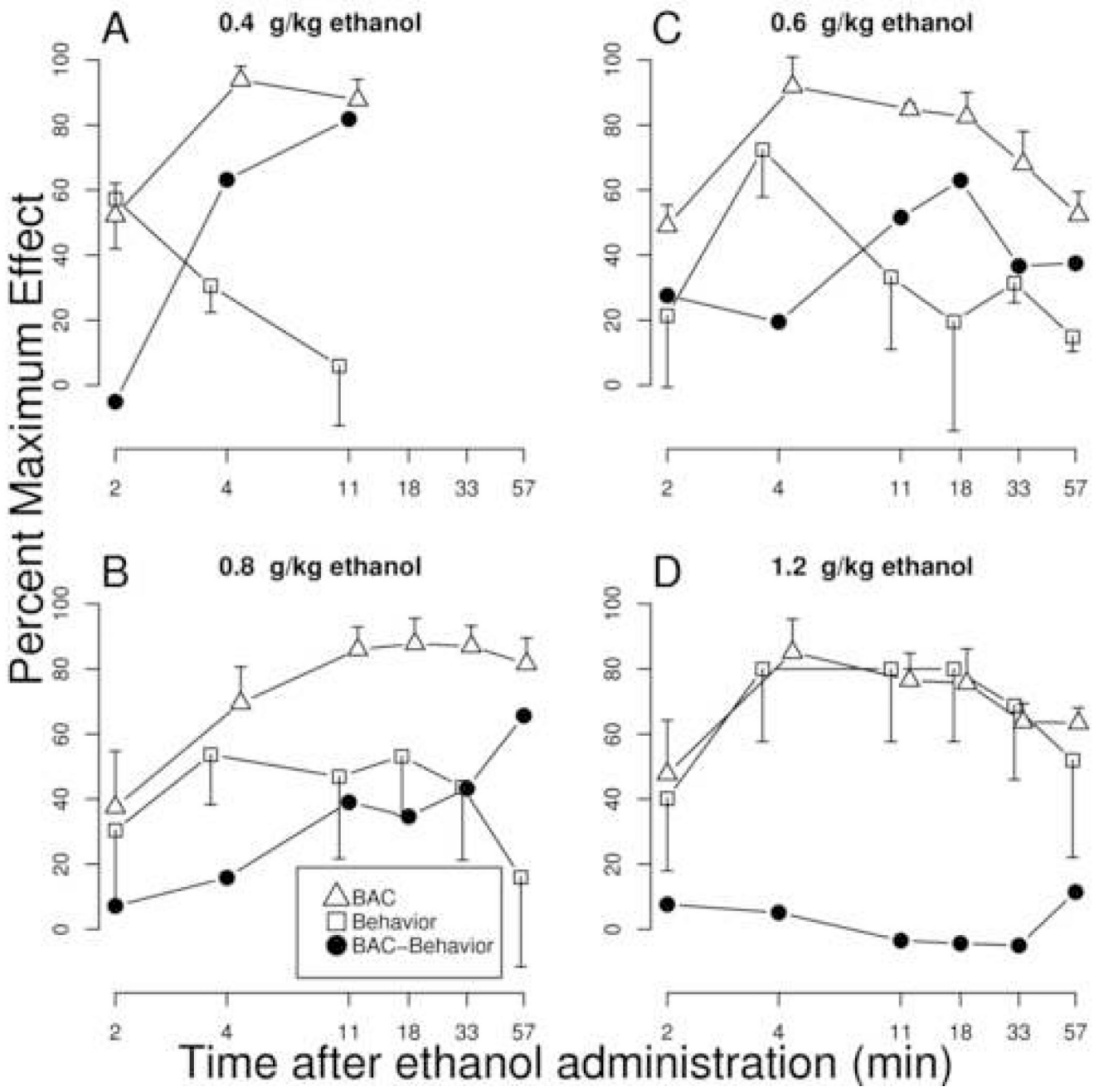

Figure 2.

Results of slope function analyses of acute tolerance to single doses of ethanol over time. Points represent the BAC and behavioral impairment (expressed as percent maximum) over time following each dose of ethanol, and reflect the mean ± S.E.M. for all 5 rats. Ethanol was administered at time zero. Slope function was calculated by subtracting the percent maximum BAC from percent maximum behavioral effect (BAC-behavior). The slope of this function was determined by performing a linear regression from the time of maximal BAC to 57-min (*see* Table 2). Points have been offset for clarity.

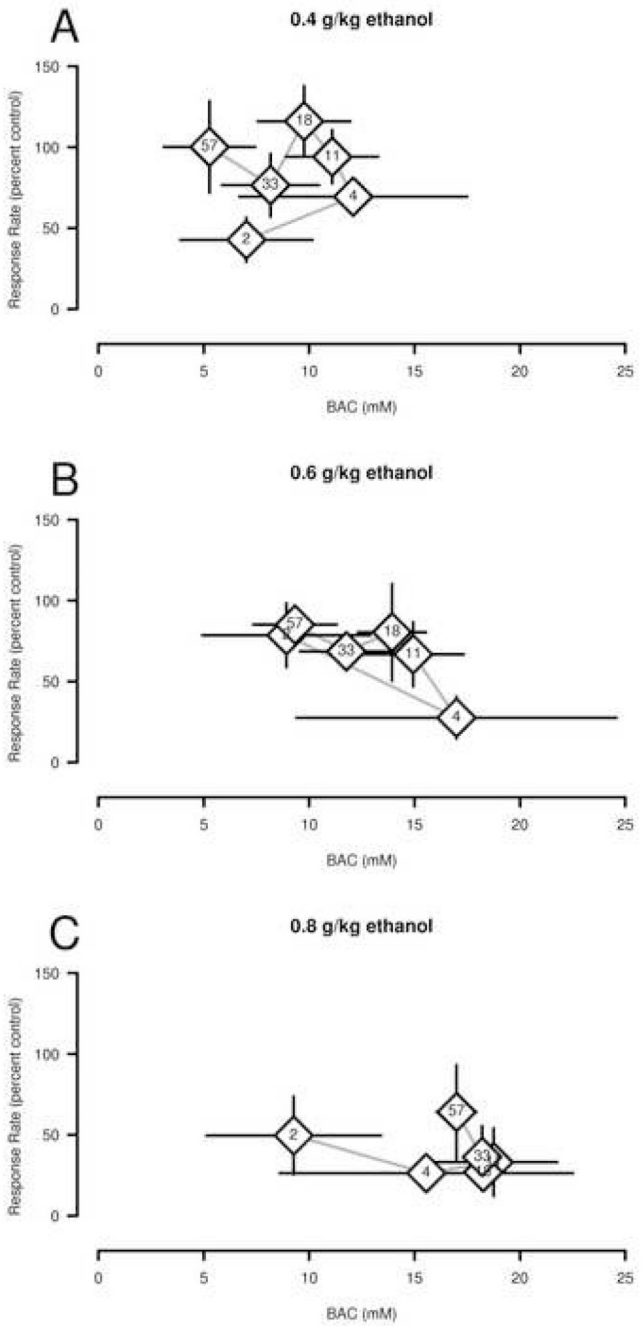

Figure 3.

Hysteresis plots of BAC and behavioral effects of each ethanol dose across time. The behavioral effect (as percent control response rate) is plotted as a function of BAC at the corresponding time point for the group data. Each point is labeled to show the time point it represents. Points represent the mean of 5 rats. Vertical bars represent S.E.M. of response rate and horizontal bars represent the S.E.M for BAC at each time point following the appropriate ethanol dose. Vertical movement with increasing time suggests acute tolerance, as response rate is recovering while BAC remains the same in this condition.

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Table 1

Area under the curve (AUC) measure summary.

		Ascending AUC		Descending AUC		Ratios (Behavior/BAC)		C		
SUBJECT	**DOSE**	**BAC**	**Behavior**	**BAC**	**Behavior**	**Ascending**	**Descending**	**(see Eq.1)**	**p<**	**adjusted p<**
1	**0.4**	32.32	166.23	26.26	181.81	5.14	6.92	**1.35**		
2	**0.4**	8.48	153.60	4.07	112.31	18.11	27.59	**1.52**		
3	**0.4**	32.34	221.53	12.58	209.13	6.85	16.62	**2.43**		
4	**0.4**	35.12	201.33	13.87	148.49	5.73	10.70	**1.87**		
5	**0.4**	19.24	103.19	13.10	135.20	5.36	10.32	**1.92**		
GROUP		25.50	169.18	13.98	157.39	8.24	14.43	**1.75**	0.01	0.05
1	0.6	13.32	146.19	11.53	102.47	10.98	8.89	0.81		
2	**0.6**	32.42	65.31	18.62	137.76	2.01	7.40	**3.67**		
3	**0.6**	41.83	88.37	29.44	171.01	2.11	5.81	**2.75**		
4	**0.6**	54.25	208.74	24.33	136.21	3.85	5.60	**1.46**		
5	**0.6**	32.53	99.49	17.82	66.09	3.06	3.71	**1.21**		
GROUP		34.87	121.62	20.35	122.71	4.40	6.28	1.43	0.14	0.28
1	**0.8**	19.47	40.22	51.65	195.00	2.07	3.78	**1.83**		
2	0.8	6.70	30.58	40.06	8.28	4.56	0.21	0.05		
3	0.8	8.89	0.00	37.71	0.00	0.00	0.00	N.D.		
4	**0.8**	18.20	73.17	45.50	186.25	4.02	4.09	**1.02**		
5	0.8	37.85	103.60	61.50	69.87	2.74	1.14	0.42		
GROUP		18.22	49.52	47.28	91.88	2.68	1.84	0.69	0.68	0.90
1	**1.2**	74.33	40.49	45.20	67.27	0.54	1.49	**2.73**		
2	**1.2**	28.47	3.78	26.94	4.27	0.13	0.16	**1.19**		
3	1.2	80.33	0.00	54.96	0.00	0.00	0.00	N.D.		
4	1.2	118.60	10.24	55.68	0.00	0.09	0.00	0.00		
5	1.2	54.42	54.93	30.67	0.00	1.01	0.00	0.00		
GROUP		71.23	21.89	42.69	14.31	0.35	0.33	0.93	0.98	0.98

N.D. - indicates value not determined due to Ascending Limb AUC = 0

BOLD - indicates entries with C > 1

Table 2

Slope function measure of acute tolerance

Ethanol Dose (g/kg)	Slope	95% Confidence Limits	r
0.4	7.83	[1.74 – 13.92]	0.75
0.6	0.13	[−0.78 – 1.04]	0.18
0.8	0.90	[−0.16 – 1.96]	0.37
1.2	0.08	[−1.13 – 1.29]	−0.03

Table 3

BAC A50 for response rate

Time (min)	A50 (mM)	95% Confidence Limits
2	2.88	(1.67–4.97)
4	6.58	(0.50–86.3)
11	22.05	(2.26–214.84)
18	32.12	(6.44–160.16)
33	23.7	(8.95–62.73)
57	0.2	(0–36.61)