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Mouse Breathalyzer

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Abstract

Background—The development of a relatively simple, noninvasive method for estimating blood ethanol concentrations in mice will be useful in behavioral studies related to alcoholism. This study validated such a method.

Methods—The apparatus consists of a body chamber fitted with a head stock through which the mouse head protrudes. This was fitted against a water-jacketed head-space chamber surrounding the mouse's head. Rebreathed air maintained at 37°C in the head-space chamber was removed using a peristaltic pump and loaded into a 1-ml injection loop. Ethanol in the sample was quantified using gas chromatography. To validate this method, ethanol levels in breath samples were compared against those in tail blood samples collected immediately after the breath samples. Breath samples were collected at 5, 10, 20, 40, 80, 120, and 160 minutes after ethanol (0.4, 0.8, 1.2, 1.6, 2.4, and 3.2 g/kg) was administered to male C57BL/6J mice.

Results—Breath and blood ethanol levels were well correlated ($r^2 = 0.96$) across time points on the descending ethanol–time curve at doses below 2.4 g/kg. Correlation for these doses on the ascending portion of the curve had greater variance, but was still well correlated ($r^2 = 0.92$).

Conclusions—The mouse breathalyzer is an accurate, convenient, noninvasive and well-tolerated method for estimating blood ethanol concentrations in mice across a range of behaviorally relevant concentrations below 2.4 g/kg, especially on the descending limb of the ethanol–time curve. Although this procedure requires a gas chromatograph in the animal facility, the ability to estimate ethanol concentrations quickly and easily will be especially useful in behavioral studies where repeated blood sampling is not feasible.

Keywords

Blood ethanol concentration; Blood alcohol concentration; Breath ethanol; Noninvasive; Alcohol

Several methods have been developed to estimate blood ethanol content noninvasively in rats (Giles et al., 1989; Hiltunen et al., 1989; Javors et al., 2005; Pohorecky and Brick, 1982). Such techniques have proven useful in studies where repeated blood sampling is not feasible (Ginsburg et al., 2005; Hiltunen et al., 1989). Despite greater difficulties in obtaining repeated blood samples in mice compared with rats, few reports have applied similar techniques in mice.

Fioriglio et al. (1980) administered radioactive ethanol to mice. Mice were placed in a metabolic chamber with an inlet and outlet for air flow. Expired air passed through the outlet and was trapped in a culture tube containing an ethanol-acetaldehyde mixture immersed in a chloroform/liquid nitrogen slurry maintained at -73°C . This procedure provides a means of determining relative levels of expired ethanol and acetaldehyde. However, the authors administered only 1 dose (3.8 g/kg) of ethanol, and did not provide a comparison of expired radioactive ethanol and acetaldehyde with blood levels. Further, the equipment used, including liquid nitrogen and radioactive ethanol present special challenges for laboratories not equipped to deal with such agents.

Thurman et al. (1982) described a method in which mice were forced to breathe in a 2.75-ml sealed breathing chamber for 17 seconds. After this equilibration period, 1 ml of air from the chamber was injected onto a gas chromatograph (GC) and the ethanol in the sample was quantified. Although the authors report a linear relationship between blood and breath ethanol concentrations, no data are presented, and no information is given on the derived linear equation or correlation. This method is potentially complicated by differences in body volume and air temperature within the sealed chamber among different subjects. Further, it is unclear if the linear relationship described applies to various doses, various time points, or both.

Another group has published an innovative technique in which ethanol vapor from the lacrimal fluid is quantified and compared with blood ethanol measures in mice (Giles et al., 1989). In this procedure, ethanol vapor is collected in a 0.6-ml plastic pipet tip held over the eye for 15 seconds. The gas in the collection vial is then sampled by vacuum transferring it onto a modified electrochemical fuel cell detector coupled with a standard voltmeter. Mice in this study were restrained in a plastic cone. Following a single dose of ethanol (1.5 g/kg), a linear relationship was present between blood and lacrimal ethanol vapor across repeated samples over time points from 0.75 to 2.5 hours after administration. This procedure is elegant; however, it is unclear whether lacrimal ethanol vapor accurately reflects central ethanol levels on the ascending limb of the ethanol–time curve.

In this study, a modified version of a previously described method for obtaining estimates of blood ethanol levels in rats using rebreathed air in a head space chamber has been applied in mice (Hiltunen et al., 1989; Javors et al., 2005; Pohorecky and Brick, 1982). The present procedure provides several advantages over previous techniques in mice. First, the relationship between blood and breath ethanol is demonstrably linear across several doses and time points, providing adequate confidence and sensitivity to quantify blood ethanol levels resulting from a wide range of behaviorally active ethanol doses. Further, no special agents (radioactive, biological, or chemical) are required, providing a technique that is useful in laboratories not equipped for these hazards. Finally, breath ethanol levels measured using this procedure likely reflect brain levels on both the ascending and descending limbs of the ethanol–time curve.

METHODS

Subjects

Male C57/BL6J mice ($n = 28$, average age on experimental day: 87 ± 8 days) obtained from Jackson Labs, Bar Harbor, ME.

Breath Samples

A restraint device was built by Institutional Instrumentation Services (UTHSCSA, San Antonio, TX). As shown in Fig. 1, a head chamber was constructed with a volume of approximately 10 cc. This chamber was jacketed inside a second chamber through which a

reciprocating pump circulated water heated to 37°C. A channel through the jacket allowed a piece of 1/16" (outer diameter) polyethyleneketone tubing to pass from the head chamber to an injection port on a GC (Thermo-Finnegan Trace GC, Waltham, MA). The body chamber consisted of a hemi-cylindrical shell that could be placed over the body and a head stock with rounded edges to restrain the mouse's head in place. A mouse's neck was easily lowered into the bottom portion of the head stock, and then the top portion was secured with the thumb screw so that any size mouse could be restrained without impeding breathing. Once the mouse was placed in the head stock with his head extended, the restraint could be fitted against the head chamber so that the mouse's head was inside. Mice were allowed to breathe in the head chamber for a set period of time, and then a sample of air from the head chamber was withdrawn for 6 seconds at a flow rate of 17 ml/min using a peristaltic pump (Gilson Minipuls2, Middleton, WI). The sample was loaded into a 1-ml stainless steel loop, then immediately injected onto a Restek Rtx[®] BAC1 column (State College, PA). The temperature of the oven, sample loop and injector, injector port, and flame ionization detector (FID) were 50°, 50°, 200°, and 250°C, respectively. The carrier gas was helium at a flow rate of 2.4 ml/min. For these experiments, the GC apparatus was set so that a 7:1 split of the sample occurred (i.e., 125 μ l of the 1-ml sample actually entered the column, and the remainder of the sample was shunted to waste). Following chromatographic separation, ethanol in the sample was quantified using flame ionization detection, data were collected, and peak areas integrated with Millenium software (Waters, Milford, MA).

Blood Samples

Immediately after each breath sample, blood (approximately 20 μ l) was collected from a nick at the end of the mouse's tail into a heparinized capillary tube. From this sample 5 μ l was pipetted into 100 μ l of 0.0025% n-propanol in water. This solution was mixed and frozen at -80°C until analyzed. On the day of the assay, samples were thawed and 1 μ l of the solution was analyzed using GC and FID detection at the Bexar County Forensic Science Center. An Agilent 6890 (Agilent Technologies, Inc., Santa Clara, CA) and Restek Rtx[®] BAC-1 column were used to facilitate the direct injection analysis. The isothermal run time was 3 minutes at 40°C. The carrier gas was helium at 11.2 ml/min, and the injector and detector temperatures were fixed at 200 and 225°C, respectively. Results were obtained utilizing GC ChemStation software version A.07.01 (Agilent Technologies, Inc., Santa Clara, CA).

Equilibration

An experiment was conducted to optimize the time that the mouse was allowed to breathe in the head chamber in order to maximize the stability of the breath ethanol levels. Four mice were injected with 1.6 g/kg ethanol. During the period of 20 to 60 minutes after ethanol administration, breath samples were taken after each mouse was allowed to breathe in the chamber for 18, 24, 30, 36, and 42 seconds. Preliminary experiments suggested that breath ethanol was relatively stable between 20 and 60 minutes after administration of this dose. The order of equilibration times (ascending or descending) was counterbalanced in these mice. Because area under the ethanol peak following 30-second equilibration was significantly greater ($p < 0.05$) from results following 18 seconds, but not different from any of the longer equilibration times, mice involved in the subsequent correlation studies were allowed a 30-second equilibration period in the head chamber.

Correlation Between Breath and Blood Ethanol Levels

Twenty-four mice were assigned to 1 of 6 groups ($n = 4$ per group). Each mouse was drug naïve prior to the study, and each mouse received only 1 dose of ethanol on the day of the experiment. Following baseline breath and blood sample collection, a dose of ethanol (0.4, 0.8, 1.2, 1.6, 2.4, or 3.2 g/kg, i.p.) was administered. Breath samples were collected 5, 10,

20, 40, 60, 80, 120, and 160 minutes after ethanol administration. Blood samples were collected immediately after each breath sample was injected into the GC.

Statistics

The correlation between blood and breath levels was performed using each blood/breath sample pair on the descending limb of the blood–time curve (time points ≥ 20 minutes postinjection). This was performed to reduce variance associated with underestimation of central blood ethanol levels by ethanol levels in the tail vein on the ascending portion of this curve (see Nurmi et al., 1999). Analysis of the curve including all points after ethanol administration made the slope slightly (but significantly) steeper and increased the variance slightly. This correlation performed on all of the data collected, and then a second time with data limited to doses below 2.4 g/kg, as mice receiving 2.4 or 3.2 g/kg displayed variable breathalyzer results (see Results) that resulted in points that were significant outliers from the global regression.

Calculation of Elimination Rates

A linear regression was performed on blood ethanol levels (converted to mg/dl) over time for each dose. Time was limited to the range of points on the descending limb from the point of peak ethanol content (20 minutes for each dose) to the last point at which ethanol content was greater than 5% of the maximum concentration observed for the group at each dose (40, 80, 120, and 120 minutes for doses in ascending order). The slope of the linear function represented elimination of ethanol from blood expressed in mg/dl/min. Confidence intervals (99%) for each slope were also determined. In order to compare our results with previous reports, elimination rates were converted to mg/kg/h by multiplying by 600 ($10 \text{ dl/l} \times 1/\text{kg} \times 60 \text{ min/h}$).

Ethanol

Ethanol (95% w/v, Aaper Chemical Co., Sheltonville, KY) was dissolved in 0.9% saline to a concentration of 20% (v/v).

Software

All analyses and graphs were prepared using R: A language and environment for statistical computing under Mandriva Linux (R undation for Statistical Computing, Vienna, Austria). Chromatographs were obtained and integrated using Millenium software.

RESULTS

Breath Ethanol

As shown in Fig. 2A, breath ethanol (as measured by integrated peak area) rose rapidly, reaching maximal levels within 10 minutes of administration. The maximal levels achieved were dose-dependent. Ethanol elimination was similar following each dose, as evidenced by slopes on the descending limb (see Table 1). Due to variability in breathalyzer results at the highest 2 doses tested, individual subject data are plotted separately for these doses in Fig. 3.

Blood Ethanol

As shown in Fig. 2B, ethanol in the blood rose more slowly than ethanol in the breath, reaching maximal levels at approximately 20 minutes after administration. Elimination of ethanol was similar to the results seen in breath samples. To allow comparison between individual breathalyzer results and blood ethanol results for the highest 2 doses, blood ethanol concentrations for individual subjects are plotted in Fig. 3 for 2.4 and 3.2 g/kg.

Correlation

Expressing breath ethanol levels as a function of blood ethanol levels collapsed across dose and time resulted in a linear relationship. The relationship for all doses and time points (Fig. 4A) is $Y = (5.89 \times 10^{-7})X + 0.030$, with $r^2 = 0.58$. When only doses lower than 2.4 g/kg are included (Fig. 4B, solid gray line), the function is $Y = (5.93 \times 10^{-7})X + 0.006$ with $r^2 = 0.84$. When only points on the ascending phase (Fig 4B, open circles and dashed gray line) of the ethanol–time curve are included, the function is $Y = (7.61 \times 10^{-7})X - 0.001$, with $r^2 = 0.92$. Finally, performing a regression on points on the descending limb (Fig. 4B, filled circles and black line) of the time curve for doses lower than 2.4 g/kg yields the equation $Y = (5.09 \times 10^{-7})X + 0.002$, with $r^2 = 0.96$. The slope for the regression including only the ascending time points following doses <2.4 g/kg was significantly different from the other calculated slopes, including the slope of the regression through all doses and time points ($p < 0.05$). None of the other slopes differed.

DISCUSSION

This study demonstrates that estimation of blood ethanol levels in mice using rebreathed air is feasible. The correlation between ethanol in tail blood and breath samples is very good ($r^2 = 0.96$) on the descending limb of the ethanol–time curve at doses below 2.4 g/kg. Adding in points on the ascending limb reduced the correlation coefficient, but still yielded a good correlation, implying that tail blood does not accurately reflect central ethanol levels on the ascending limb of the curve in agreement with Nurmi et al. (1999). Finally, the relationship between breath and blood ethanol levels is poor at the highest 2 doses tested (2.4 and 3.2 g/kg). This is due to variability in the breath ethanol measures, and likely the result of ethanol effects on respiration rate. The overall correlation coefficient between breath and blood levels reflects this discrepancy.

Considering the relationship between breath and blood ethanol levels on the descending limb of the curve for doses up to and including 1.6 g/kg, the correlation was reliable from a maximal blood ethanol concentration of 0.17 g/dl to complete elimination of ethanol from the blood, encompassing a range well below behaviorally active levels to those well above behaviorally impairing levels. Thus, this procedure seems well suited for investigations involving behavioral effects of ethanol up to and including 1.6 g/kg. Further, this technique is noninvasive. Repeated sampling is not limited due to blood access or altered blood volume. Additionally, blood volume remains constant throughout the sampling period, in contrast with procedures that require blood sample removal. Mice can easily be habituated to the procedure, allowing estimation of blood ethanol levels during behavioral tasks. Indeed, the limit of detection with the present method would allow quantification of expected blood ethanol levels after self-administration or drug discrimination procedures in mice (Becker and Baros, 2006; Gentry et al., 1983).

The results of this study are consistent. Both breath and blood ethanol levels displayed dose- and time-dependent changes as expected. Individual variation was not significant at lower doses and did not contribute to the variance of the group results. However, as noted above, breath ethanol levels following higher doses were variable, and did contribute to the group variance.

Maximum blood ethanol levels ranged from 0.03 g/dl following 0.4 g/kg to 0.17 g/dl following 1.6 g/kg. The elimination rate for each dose is shown in Table 1. These elimination rates (for both blood and breath ethanol concentrations) are similar to those reported in blood by Schlesinger et al. (1967), which ranged from 524 to 668 mg/kg/min in C57BL/6J mice, or by Abel (1982) as cited in Livy et al. (2003) who reported an elimination rate of 550 mg/kg/min in mice.

Blood ethanol levels reached a maximum more slowly than breath ethanol levels. This is not surprising considering that blood was collected from the tip of the tail, and the reduced perfusion in the tail has been shown to result in underestimation of brain and trunk ethanol levels on the ascending portion of the ethanol–time curve in rats (Nurmi et al., 1999). However, elimination of ethanol was similar in blood and breath samples.

In our previous studies in rats, blood sampled from the vena cava provided a better correlation with breath ethanol levels on the ascending limb of the curve (Javors et al., 2005). This is expected based on the low perfusion of blood into the tip of the tail compared with venous blood in the trunk (Nurmi et al., 1999). Indeed, in rats, the conversion factor to calculate blood levels from breath levels was the same on both limbs of the curve (Javors et al., 2005). Therefore, based on our previous results in rats using a similar apparatus, and based on the breath ethanol levels seen in mice in the present study, it is highly likely that this method provides an accurate assessment of blood ethanol levels on the ascending portion of the curve.

This procedure should prove useful in a variety of behavioral experiments. Studies of behaviors following rather low doses of ethanol such as ethanol discrimination and self-administration could apply this procedure to determine blood ethanol levels. Application of this procedure may also be useful in studies comparing different phenotypic responses to ethanol.

Still, it must be noted that this procedure is not applicable to behavioral measures that require higher doses of ethanol (above 1.6 g/kg). The variance in breath ethanol levels seen at higher doses is consistent with the effects of respiratory depression expected at these doses. Respiratory depression would slow equilibration of ethanol in the head chamber. Thus, breath ethanol variance at these higher doses might be reduced with a longer equilibration time, which would allow this method to be used in studies requiring higher dose. However, additional experiments are necessary to verify this notion.

In conclusion, this procedure appears to provide reliable estimation of blood ethanol levels in mice, noninvasively. The relationship between blood and breath ethanol levels is linear across a wide range of blood ethanol values. This allows reliable estimation of blood levels relevant to a variety of behavioral measures of ethanol's effects. The procedure appears to be well tolerated by mice. Thus, we report a technique that is noninvasive, reliable, sensitive, and thus well suited for estimating blood ethanol levels in behavioral studies of ethanol effects in mice.

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Fig. 1. Illustration of the mouse breathalyzer apparatus. Mice are placed into the lower part of the head stock and the upper portion is placed so that the subject can breathe normally, but cannot withdraw its head. The thumb screw secures the stock in place. The body chamber is secured by a thumb screw to keep the subject's body in line with the head. The mouse is then placed so that his head is completely inside the head chamber. The water jacket maintains the head chamber at 37°C. A tunnel through the water jacket allows tubing from the injection loop to enter the head chamber for sampling.

Fig. 2. Time course of ethanol levels in breath (**A**) and blood (**B**) following ethanol administration at zero minutes in mice ($n = 4$ per dose). Points represent mean levels expressed as integrated peak area in breath or g/dl in blood. Error bars represent the SEM.

Fig. 3. Time course of ethanol levels in breath (A,C) and blood (B,D) following 2.4 g/kg (A,B) or 3.2 g/kg (C,D) ethanol administration at zero minutes in mice ($n = 4$ per dose). Each symbol represents an individual mouse at a given dose.

Fig. 4.

Breath ethanol levels expressed as a function of blood ethanol concentration and the resulting correlation. **(A)** Data for all doses of ethanol. Gray circles are reproduced from panel A (doses <2.4 g/kg), downward triangles are points following 2.4 g/kg, and upright triangles are points following 3.2 g/kg. The gray line is the regression through all the data points. **(B)** Data for doses less up to and including 1.6 g/kg. Each point represents a pair of breath and blood ethanol samples at 1 time point following 1 dose of ethanol in 1 subject. Open circles represent points on the ascending limb of the ethanol–time curve, while black circles represent points on the descending limb of the ethanol–time curve. The solid gray line is the regression through all the points, the dashed gray line is the regression through points on the ascending limb, and the solid black line is the regression for points on the descending limb.

Table 1

Blood Ethanol Elimination Rates

Ethanol dose (g/kg)	Elimination rate (mg/dl/min)	99% CI	r^2	Elimination rate (mg/kg/h)
0.4	-0.74	(-1.35, 0.11)	0.76	-441.6
0.8	-1.15	(-1.56, -0.74)	0.90	-689.4
1.2	-1.16	(-1.73, -0.60)	0.73	-698.4
1.6	-1.13	(-1.36, -0.89)	0.91	-675.6
2.4	-0.80	(-1.10, -0.51)	0.73	-481.8
3.2	-0.71	(-1.04, -0.38)	0.63	-426.282